Stem Cell Marker OCT3/4 in Biology and Diagnostics of Germ Cell Tumors

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Coverdrawing by Anoek & Jeroen de Jong, detail of the front page from “Exercitationes de Generatione Animalium” (1651) by William Harvey.

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Stem Cell Marker OCT3/4 in Biology and Diagnostics of Germ Cell Tumors

Stamcel marker OCT3/4 in de biologie en diagnostiek van kiemceltumoren

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"*Ik hoop dat ik het nog mag meemaken, jongen.*"

Ter nagedachtenis aan opa Ouweschoen

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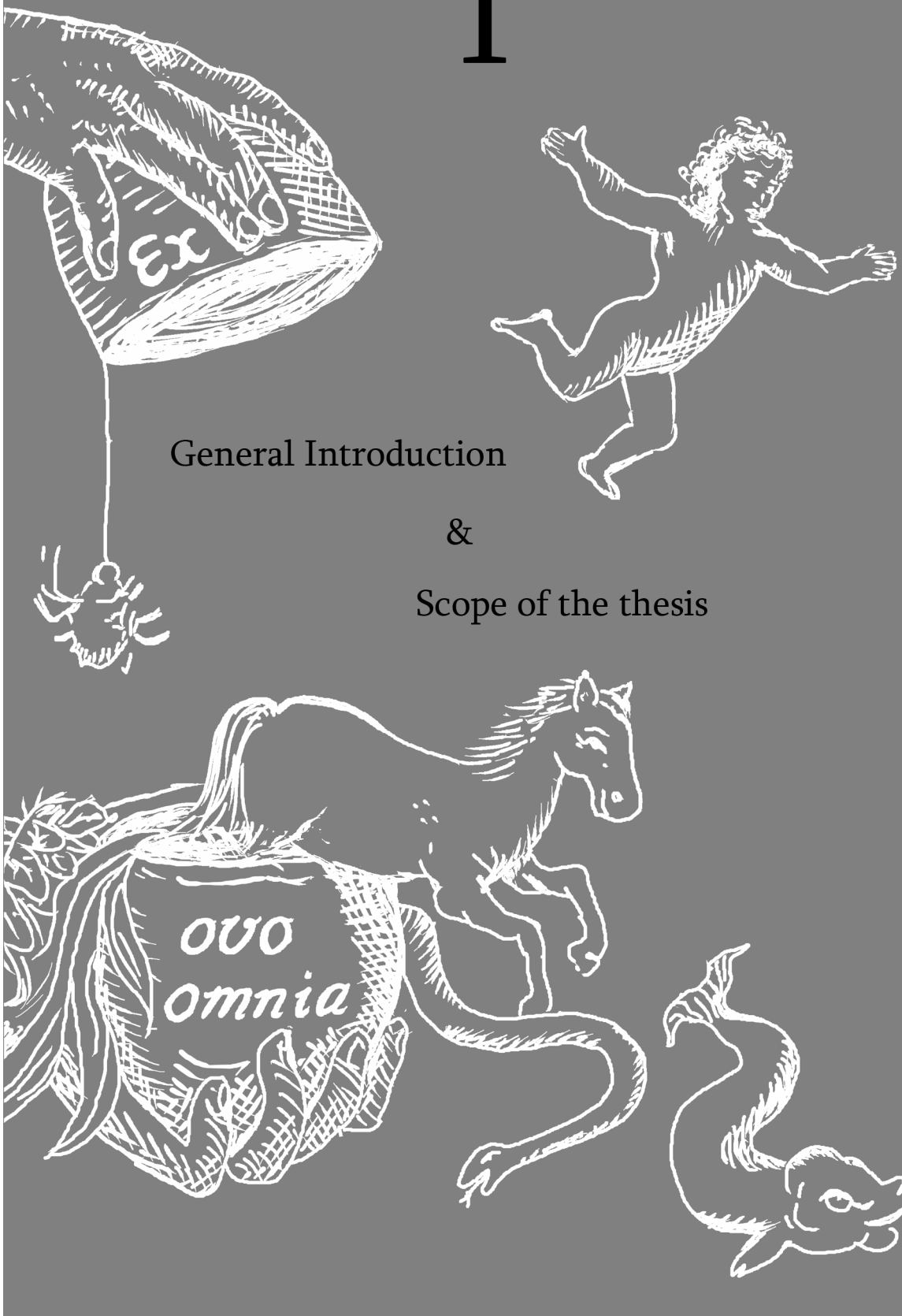

General Introduction

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Scope of the thesis

Adapted from:

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INTRODUCTION

Pluripotency is regarded as the hallmark of embryonic stem (ES) cells. The ability of these cells to differentiate into all three germ layers during embryogenesis, which give rise to specialized tissues and organs, is the requisite to form complex multi-cellular organisms. In addition, this pluripotency is transmitted through the germ line to the next generation. Although the concept of pluripotency was already implied in William Harvey's postulation "Ex ovo omnia" in the 17th century, the mechanisms regulating pluripotency on the molecular level have only been partly elucidated nowadays. Based on results from the past 17 years, transcription factor OCT3/4 is currently regarded as one of the key regulators of pluripotency.[1,2] The central role of OCT3/4 in keeping cells in an undifferentiated pluripotent state and preventing differentiation has been a major focus in stem cell research. In germ cell development prolonged expression of OCT3/4 is tightly linked to malignant transformation and the genesis of germ cell tumors of adolescents and young adults. A detailed overview of the role of OCT3/4 during normal embryogenesis is provided and translated to its current status in the study of germ cell tumorigenesis.

HISTORY AND STRUCTURAL TRAITS

Initially, OCT3/4 was identified in nuclear extracts of undifferentiated cells of the murine embryonal carcinoma (EC) cell line F9 and designated nuclear factor A3 (NF-A3).[3] Subsequently, different names have been used for this protein as OCT3[4,5], OCT4[6,7] and a combination of the previous, OCT3/4[8].

The OCT3/4 transcription factor belongs to a family of octamer binding proteins that specifically bind to the conserved ATTTGCAT motive in transcriptional control elements of genes.[9] The octamer motive is recognized by the highly charged POU domain[10] in the OCT3/4 protein, from which the gene name *POU5F1* (POU family of transcription factors, class 5, factor 1) is derived. On the DNA level the POU domain consists of two subdomains: a C-terminal homeodomain and an N-terminal POU-specific region separated by a short non-conserved linker.[11] The human *POU5F1* gene has been mapped to chromosome 6p21 and consists of 5 exons from which two alternative splice variants can be transcribed.[12,13] In most reports, only the long splice variant, isoform A, is studied, however recently there has been increased interest in the short splice variant, isoform B.[14-16] This last isoform is localized in the cytoplasm and is unable to activate or repress transcription of OCT3/4 responsive genes in contrast to isoform A.[15] Because of a polymorphism at the initiating codon, not all individuals can form the isoform B protein.[12] Therefore, the biological function of the human OCT3/4 isoform B mRNA and protein remain to be established.

Next to the *POU5F1* gene six pseudogenes have been identified in the human genome.[17,18] These pseudogenes can be transcribed, but a protein will never be formed.

FUNCTION OF OCT3/4 EXPRESSION IS CELL TYPE DEPENDENT

The presence of OCT3/4 is vital for the formation of pluripotent stem cells during embryogenesis as shown in a mouse knock-out study by Nichols *et al.*[19] OCT3/4 deficient embryos develop to blastocyst-like structures with a normal cell number at the site of the prospective inner cell mass (ICM), but after implantation no homozygous mutant embryos are found. The ICM cells of these embryos are not pluripotent and are restricted to differentiation along the extra-embryonic trophoblast lineage during *in vitro* propagation.[19] Tightly regulated expression levels of OCT3/4 dictate pluripotency and differentiation. In mouse ES cells with inducible OCT3/4 regulation, Niwa *et al* [20] showed that a 50% decrease in expression levels resulted in trophectodermal differentiation, whereas a 50% increase initiated upregulation of primitive endoderm and mesoderm markers. This confirms the previous results from Palmieri *et al* [21] who described OCT3/4 protein expression to appear higher in the newly forming primitive endoderm than that seen in the ICM population of murine blastocysts. In addition, Botquin *et al* [22] found a transient 2.5 fold increase in OCT3/4 mRNA levels after 1 day of retinoic acid induced differentiation of murine EC cells through a hypoblast-like stage. Differentiation along the trophectodermal lineage as a result of OCT3/4 downregulation was recently also shown by RNA interference (RNAi) targeting the OCT3/4 mRNA in mouse and human ES cell lines.[23-26] Besides expression in undifferentiated cell types in early development, OCT3/4 is also present in migrating primordial germ cells (PGCs). These cells are not pluripotent *in vivo* but committed to gametogenesis after they reach the developing gonads. Conditional gene targeting during murine PGC migration indicates that loss of OCT3/4 presence leads to apoptosis instead of differentiation to trophectoderm.[27] This suggests that OCT3/4 has a function in survival of these cells during migration. However, conditional gene targeting by the same strategy as *in vivo* in cultured embryonic germ cells derived from 8.5dpc PGCs leads to trophectodermal differentiation instead of apoptosis.[27] This is consistent with the observation that cultured human PGCs can give rise to pluripotent stem cells capable of forming embryonic bodies that contain cells of all three germ layers.[28] How the function OCT3/4 exerts in a certain cell type is intrinsic to that cell or is dependent on its niche remains to explored further. The mechanism and pathway by which OCT3/4 promotes survival in PGCs and how this is linked to its role in pluripotency is unknown at the moment.

ROLE IN NORMAL DEVELOPMENT

The role of OCT3/4 in early mammalian development has first been recognized in the mouse[4,5,7,29] and was subsequently studied in human[14,30,31], bovine[32,33], porcine[33], marsupial[34] and monkey[35] embryogenesis. In general, expression patterns are the same in all mammals, however there are some marked and significant differences, especially in the early stages of embryogenesis and gametogenesis. The interspecies differences in early embryogenesis could be expected because of species-specific timing of activation of embryonic transcription when transition from oogenetic to embryonic control of

development is initiated and the cells of the embryo begins to synthesize their own mRNA and protein, thereby replacing the mRNA and protein pool maternally inherited from the oocyte.[36,37] Besides the variation between species, there are also inconsistencies between studies on the same animal.

Expression of OCT3/4 in human embryogenesis was first reported in 1995 by Abdel-Rahman *et al* [30] using murine-based degenerate primers for RT-PCR on embryos at the 1-cell to blastocyst stages. These embryos were not suitable for assisted reproduction because of retarded development and were therefore available for research. Throughout development, from unfertilized oocyte to blastocyst, OCT3/4 expression was detected. To clarify the distribution pattern of OCT3/4 in human blastocysts, Hansis *et al* [31] examined mRNA expression in ICM and trophectoderm cells of blastocysts discarded for *in vitro* fertilization due to anomalous morphology. Blastocysts were halved, creating a trophectoderm part and a mixed ICM/trophectoderm part and the OCT3/4 expression levels were found to be significantly higher in the ICM/trophectoderm compared to the trophectoderm samples. In addition, the size of the ICM was well correlated with the OCT3/4 mRNA levels, indicating a rather similar level in each individual ICM cell. Recently, Cauffman *et al* [14] performed a large study on OCT3/4 mRNA and protein expression in a series of 36 oocytes of various maturity and 112 normally developing pre-implantation embryos at the level of single blastomeres, morulas, blastocysts, or ICM and trophectoderm samples. Oocytes and cleavage stage embryos revealed a variable expression pattern, concomitant with a pure cytoplasmic localization of the protein. During compaction, the variability in expression faded away indicating embryonic OCT3/4 expression and the protein appeared in the nucleus implying biological activity. In blastocysts, transcripts and protein were present at approximately the same levels in ICM and trophectoderm, which is in contrast to the previous study by Hansis *et al* [31], expression profiling by Adjaye *et al* [38] and studies in murine embryos[21,33], but consistent with studies in bovine and porcine embryos[32,33]. In Rhesus monkey the study by Mitalipov *et al* [35] points to a position in between where early blastocysts have OCT3/4 expression in the trophectoderm that diminishes quickly as these blastocysts expand and hatch.

Immediately after implantation, it is not possible to follow the fate of OCT3/4 positive cells in human embryos. Studies in the mouse using mRNA *in situ* hybridization[5] and transgenic mice expressing green fluorescent protein under control of the OCT3/4 promoter[39], indicate that OCT3/4 expression becomes restricted to the epiblast until the beginning of gastrulation when it is downregulated in an anterior to posterior manner, after this only PGCs express OCT3/4. These cells move through the primitive streak and invade the definitive endoderm, parietal endoderm and allantois. The fate of the PGCs in the allantois is uncertain, however the PGCs from the definite endoderm are incorporated into the hindgut pocket and migrate to the developing gonadal ridges.[40] After the PGCs have reached the genital ridge, they are called gonocytes.[41] In the human embryo this stage is reached in the sixth week of development[42] and these cells have been studied from terminated pregnancies as early as 8 weeks.[43] In the developing testis these OCT3/4 positive gonocytes are initially located in the lumen, there they change ultrastructural features and move to the basal lamina, where they are called prespermatogonia, which are OCT3/4 negative.[41,44] Gradually, the number of OCT3/4 positive cells decreases with time,

although they can occasionally be detected in neonatal testes.[44] In normal human adult testis and spermatogenesis no OCT3/4 positive cells are present[45] in contrast to adult mouse testis in which Gidekel *et al* [46] detected OCT3/4 positive spermatogonia A with a self-made antibody.

In the female gonad, OCT3/4 protein expression decreases after ~24 weeks of gestation.[47] At term and in the ovaries of neonates, hardly any positive germ cells were detectable. In addition to oogonia, OCT3/4 was occasionally seen in the cytoplasm of early oocytes, but was never detectable in cells involved in folliculogenesis.[47,48]

THE PLURIPOTENCY NETWORK

Maintaining cells in an undifferentiated state requires fine-tuning of an intricate network of cellular factors that prevents the cell from entering any of the differentiation lineages. Tightly controlled expression levels of OCT3/4 have been shown essential in keeping cells undifferentiated and pluripotent.[20] Besides OCT3/4, a number of other embryonic stem cell specific proteins have been identified that contribute to the so-called "stemness" of pluripotent cells.[38,49-56] Among these are well-characterized transcription factors as NANOG and SOX2.[57-59] SOX2 (SRY-related HMG box 2) is an HMG domain-containing transcription factor that shows an expression pattern similar to OCT3/4 during early embryogenesis.[59] The HMG-domain and homeodomain DNA binding sites are frequently adjacent or in close proximity to each other and their co-evolution suggest a role for specialized SOX/POU protein complexes.[60] Indeed, for OCT3/4 and SOX2 this protein interaction during DNA binding has been confirmed.[60,61] In addition, Boyer *et al* [62,63] demonstrated by chromatin immunoprecipitation combined with DNA microarrays (ChIP-on-chip) that 65% of OCT3/4 direct downstream target genes in human ES cells are also regulated by SOX2. Comparative location analysis in mouse ES cells indicated that OCT3/4 and SOX2 targets overlap substantially.[62,63] This strengthens the functional partnership of OCT3/4 and SOX2. Among the target genes are *OCT3/4* and *SOX2* themselves and the SOX2-OCT3/4 protein complex regulates *OCT3/4* and *SOX2* gene expression.[64] Transcriptional regulation of *NANOG* gene expression is also dictated by joined OCT3/4 and SOX2 binding.[65,66] The homeobox transcription factor NANOG is required to maintain pluripotency in mouse epiblast and ES cells and RNA interference induced downregulation of NANOG in human ES cells leads to extra-embryonic endoderm and trophectoderm differentiation.[67,68] NANOG exerts its function in a close relationship to OCT3/4 and SOX2 as NANOG regulates more than 90% of the OCT3/4-SOX2 direct downstream gene targets.[62] The interdependency is further exemplified by the need for OCT3/4 and NANOG to function in parallel in maintaining ES cell self-renewal and that neither is dispensable nor capable of compensating the role of the other.[69]

Unravelling the regulation of all components in the pluripotency network is a laborious task that requires high through-put screening techniques as well as detailed functional analyses of each individual factor. Many studies have focussed on the major players OCT3/4, SOX2 and NANOG as discussed above. However, other known "stemness" factors

as REX1/ZFP42[70], CRIPTO-1/TDGF1[71], STELLAR/DPPA3[72] and ESG1/TLE1[73] are just a few examples of the many components whose functional role remains to be established.
An approach to dissect the pluripotency network is to classify each component based on its relation to OCT3/4. This generates three classes: the first containing the upstream factors that bind to the regulatory elements of the *OCT3/4* gene either activating or repressing transcription. Almost all of these factors bind in one of the four conserved regions upstream of the *OCT3/4* gene (Figure 1).[74] The second class consists of proteins physically interacting with the OCT3/4 protein and the final class is formed by downstream target genes that are directly activated or repressed by binding of OCT3/4 to the octamere DNA sequence. Of course, these classes will show some overlap because of positive and negative feedback mechanisms and proteins that can bind to OCT3/4 protein and its DNA sequence. The bulk of factors identified thus far are repressed downstream targets of OCT3/4 like transcription factors involved in developmental processes in human and murine embryogenesis.[62,63] A large screen on the physical interaction of OCT3/4 with other proteins has only been performed in murine ES cells by Wang *et al*[75]. Using affinity purification under native conditions of OCT3/4, NANOG and REX1 amongst others, followed by mass spectrometry, physically associated protein partners were identified and a protein interaction network was constructed. As OCT3/4 also regulates its own gene expression, the identified protein partners are potential upstream regulators of OCT3/4 expression either indirectly via the OCT3/4 protein, directly by binding to the DNA, or via both ways. Remarkable was the absence of the well-characterized OCT3/4 protein partner SOX2, especially since SOX2 has direct functional contact with the NANOG protein in activation of the *REX1* gene.[70] Another factor that was previously reported as a physical partner for OCT3/4 is the early trophectodermal marker CDX2 that reciprocally represses OCT3/4 target genes.[76] CDX2 was also not present in the protein interaction network.[75] In mouse embryonic stem cells, it was found that β-CATENIN, a component of the WNT signalling pathway, is required for maintenance of undifferentiated ES cells and can physically interact with OCT3/4.[77] This adds to the view of WNT signalling being involved in virtually every aspect of embryonic development and controlling homeostatic self-renewal in a number of adult tissues.[78] Besides the OCT3/4 protein partners that can indirectly influence OCT3/4 transcription, a relatively small number of transcription factors has been identified that bind directly to the transcription regulation elements of the *OCT3/4* gene. As discussed above, OCT3/4, NANOG and SOX2 are the most prominent factors during the maintenance of pluripotency. Another class of factors that has been shown to positively or negatively regulate OCT3/4 transcription, consists of the orphan nuclear receptors for which specific ligands have not yet been defined like steroidogenic factor-1 (SF1; ELP; NR5A1)[79,80], liver receptor homologue 1 (LHR1; FTF; NR5A2)[81], COUP transcription factors ARP-1, EAR-2 and EAR-3[82-84], TR2 (NR2C1)[85], and germ cell nuclear factor (GCNF; NR6A1)[86]. This last factor recruits DNA methyltransferase DNMT3A for *OCT3/4* gene silencing.[87] Nuclear receptors with a defined ligand are the retinoic acid receptors (RAR; isoforms α, β and γ) and the retinoid X receptors (RXR; isoforms α, β and γ) that have been shown to direct early embryogenesis, testis development and meiotic timing.[88-91] They bind retinoic acid and can form heterodimers. Three of these heterodimers, RARα:RXRα, RARβ:RXRα and

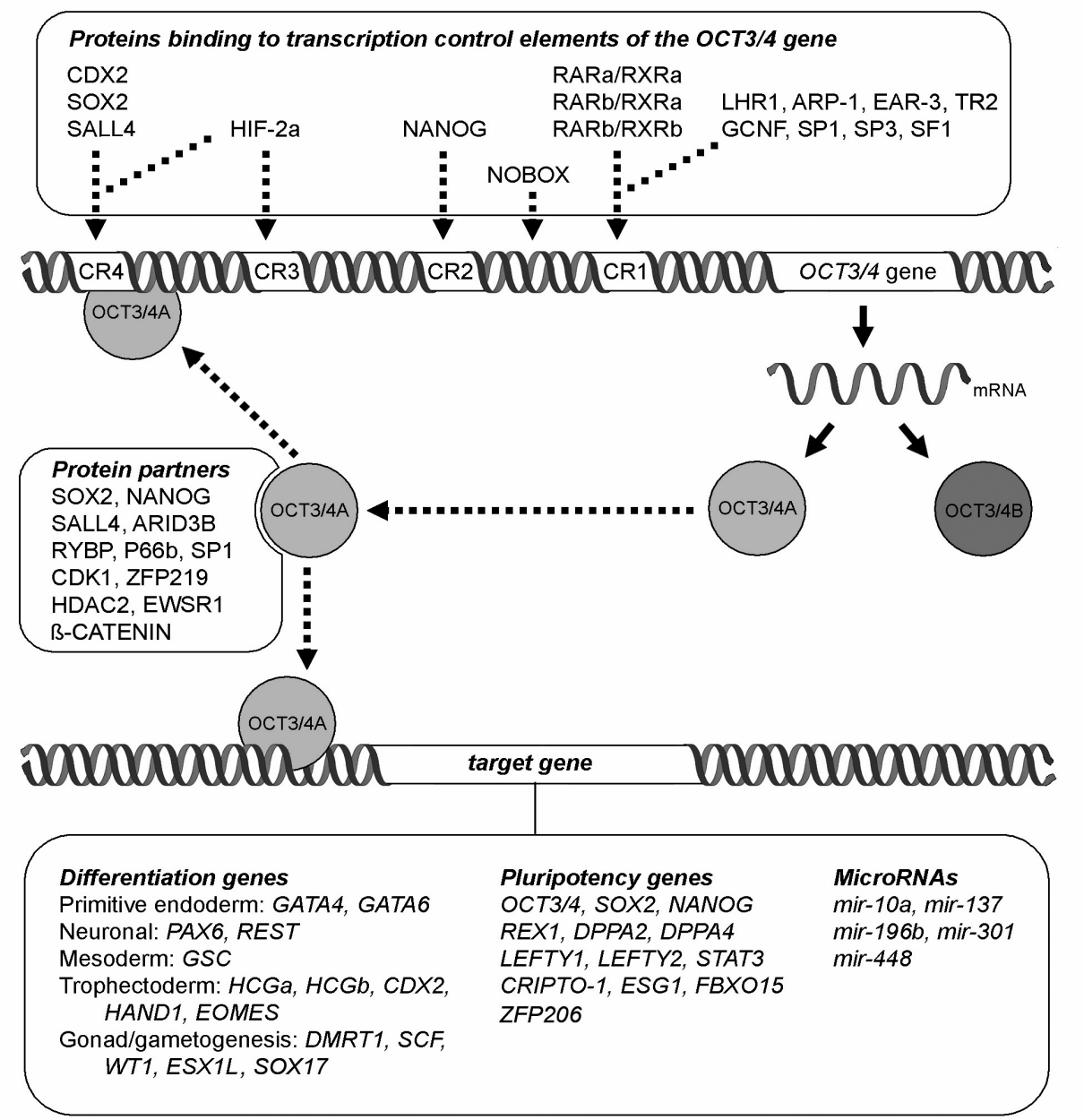

FIGURE 1. Transcriptional regulation of the *OCT3/4* gene and targets of OCT3/4 protein. For clarity the well-established name *OCT3/4* is used in this figure, however the official gene name is *POU5F1*. Transcription of the *OCT3/4* gene is regulated by a large number of proteins that bind to conserved regions (CR1 to CR4) in the upstream region of the gene. Which factors will bind depends on the stage of embryonic development, cell type and environment of the cells involved. The binding site for NOBOX (between CR1 and CR2) is only present in the murine *OCT3/4* gene, whereas, in theory, the other factors can bind in both the mouse and human gene. However, most of the functional studies have been performed in mouse and await confirmation in human cells. Activation of *OCT3/4* expression will generate mRNA that can be alternatively spliced in human generating two proteins, OCT3/4A and OCT3/4B. The biological role of OCT3/4B is unknown at the moment. The OCT3/4A protein can bind to several other proteins, but so far again most of these interactions have only been studied in mouse. In addition, OCT3/4A binds to transcriptional control elements and activates or represses expression of a large number of genes. Besides regulating its own expression by binding to conserved region four (CR4) upstream the *OCT3/4* gene, OCT3/4A regulates genes involved in differentiation and pluripotency of which a selected number from human and mouse data is presented here. Finally, OCT3/4A binds in the vicinity of a number of miRNAs and is therefore likely to influence their expression. *(Figure 1 colour image on page 177)*

RARβ:RXRβ specifically bind and activate the *OCT3/4* promoter in a ligand-dependent manner.[82,88] This stimulation is antagonized by COUP transcription factors ARP-1 and EAR-3 that down regulate *OCT3/4* promoter activity, whereas the DNA-binding specificity proteins SP1 and SP3 are also involved in this interaction.[82,83,92] Recently identified upstream regulators of OCT3/4 expression are newborn ovary homeobox gene (NOBOX)[93], hypoxia-inducible factor 2α (HIF-2α)[94] and a member of the spalt-like protein family SALL4[95]. SALL4 activates OCT3/4 expression by direct DNA binding and physical protein interaction with OCT3/4 and NANOG.[75,96] In addition, SALL4 co-occupies NANOG genomic sites in embryonic germ cells suggesting a similar NANOG-SALL4 partnership as OCT3/4-SOX2.[96]

Next to downstream target genes whose mRNA expression is under control of the OCT3/4 protein, expression of specific members of a class of small non-coding RNAs known as microRNAs (miRNAs) is likely to be regulated by OCT3/4. MiRNAs are ~22 nucleotide RNAs that use antisense complimentarity to inhibit translation of specific mRNAs to protein. Recent studies of expression patterns and functional roles have implicated specific miRNAs in complex genetic pathways regulating embryogenesis, hematopoiesis, neuronal differentiation and Hox-mediated development (see Pasquinelli *et al* [97] for review). DNA binding sites for OCT3/4, NANOG and SOX2 were found in the vicinity of a small number of miRNAs (Figure 1).[62] An additional piece of evidence for a functional relationship between OCT3/4 and expression of certain miRNAs is the co-occupation of the presumed promoter of mir-301 by OCT3/4, NANOG and SOX2 and the presence of mir-301 in human ES cells.[62,98] Finally, RNAi silencing of DICER1, an RNAse III enzyme involved in the cytoplasmic processing of miRNAs, suppresses OCT3/4 as well as NANOG and SOX2 mRNA and protein expression, and DICER1 knock-out embryos develop to the blastocyst stage with an OCT3/4 negative ICM.[99,100] In contrast, DICER-null mouse ES cells generated by conditional gene targeting express OCT3/4 at normal levels, but cannot differentiate both *in vivo* and *in vitro.*[101]

So far, mainly the nuclear factors that function in maintaining pluripotency have been identified. The extra-cellular signals and their intracellular pathways that disturb this intricate pluripotency network and lead to differentiation need further exploration. A schematic model of *OCT3/4* regulation, its protein partners and downstream target genes is presented in Figure 1. Most of the evidence of the included factors comes from murine ES cells or transgenic mice and awaits to be investigated in human experimental systems. In addition, the factors can be cell-type specific and most functional studies have focussed on ES cells; however OCT3/4 regulation and function can be substantially different in for example PGCs.

EPIGENETIC REGULATION OF THE *OCT3/4* GENE

Next to the binding of factors on transcription regulatory elements as described above, gene expression during normal development is also tightly regulated by epigenetic modifications, including cytosine (CpG) methylation and histone modification, including acetylation.[102] A model for *OCT3/4* gene silencing was recently proposed by Feldman and

co-workers[103] who described a sequence of inactivation. First, factors like GCNF bind for transient inactivation, followed by deacetylation and subsequently DNA methylation catalysed by *de novo* methyltransferases DNMT3A and possibly DNMT3B, which are recruited to the promoter through involvement of effectors as G9A and HDAC (HDAC2 is a direct binding partner of the OCT3/4 protein[75]; see Figure 1). This methylation step brings an irreversible repression of OCT3/4 expression preventing reactivation of the gene and dedifferentiation of cells.

Methylation of the *OCT3/4* upstream region has been studied in several human and murine tumor-derived and ES cell derived cell lines. In mouse ES cells, Hattori *et al*[104] showed the hypomethylation of this region and compared it to trophoblast stem cells and mouse liver cells which were hypermethylated. In addition, they showed that *in vitro* methylation suppressed *OCT3/4* enhancer/promoter activity in a reporter assay; a defect in the methylation machinery resulted in the ectopic expression of *OCT3/4*, and the demethylating agent 5-aza-2'-deoxycytidine could induce aberrant *OCT3/4* expression in trophoblast stem cells. This possibly indicates to dedifferentiation of these cells.[104,105] In cloned mouse embryos the *OCT3/4* promoter undergoes gradual demethylation during pre-implantation development and inefficient demethylation of the *OCT3/4* promoter is associated with developmental retardation at early cleavage stages.[106] In the malignant equivalent of ES cells, the embryonal carcinoma (EC) cells, it was already demonstrated in 1993 that inhibition of *OCT3/4* expression in retinoic acid-induced differentiated murine EC cells is achieved through changes in methylation status, chromatin structure and transcriptional activity of the *OCT3/4* upstream regulatory region.[107] These results were confirmed by independent studies.[8,108] Loss of *OCT3/4* expression during differentiation of a human EC-derived cell line (NTera2) was found to be correlated with increased methylation in the *OCT3/4* upstream region.[109] This correlation was also found in human ES cells.[110]

OCT3/4 IN ADULT STEM CELLS

Expression of OCT3/4 in ES cells, EC cells and PGCs was proven in multiple independent studies. A consistent triad of hypomethylation of the gene promoter region, high expression of OCT3/4 mRNA and abundant presence of the protein in the nucleus of these cells, has been reproduced repeatedly by different approaches like RT-PCR, micro-array expression profiling, Western blotting and immunohistochemistry. However, it is still a matter of debate if OCT3/4 expression is restricted to these aforementioned cells. Already in 1992, Takeda *et al* [12] reported expression of both splice variants in normal human heart, kidney, liver, spleen and placenta. It is also interesting that in this study the transcripts were picked up from a cDNA bank of adult human pancreatic islets. Recently, an accumulating pile of articles has been published reporting OCT3/4 expression in normal adult tissues and various tumors and cell lines. This increased focus on OCT3/4 has been triggered by the concept of adult stem cells, which are tissue specific stem cells with the capacity for self-renewal and tissue regeneration.[111-113] The current hypothesis is that asymmetrical division of a stem cell generates a new stem cell and a multipotent

progenitor cell, which is restricted to differentiation to cell types within a particular organ. These tissue specific stem cells or their multipotent progenitor cells can give rise to tumor formation when the homeostasis of self-renewal and tissue regeneration is disturbed either by intrinsic properties of the cells themselves, i.e. mutations, or by changes in the microenvironment, i.e. the stem cell niche.[111,112] Although progress has been made in identifying specific cell populations within tumors that can give rise to new tumors in transplantation experiments, there remains debate whether these are the true adult stem cells and how to further define and characterize them.[112,114]

A MARKER FOR GERM CELL TUMORS OF ADOLESCENTS AND ADULTS

Human germ cell tumors (GCTs) form a heterogeneous group that can mimic various stages of intra-uterine development. Recently, we proposed to classify these tumors into five subtypes based on anatomical site, phenotype, age of presentation, cell of origin, genomic imprinting pattern, genotype and animal model.[123] This classification is adopted by the World Health Organisation and includes: type I: teratomas and yolk sac tumors of neonates and infants; type II: seminomatous and nonseminomatous tumors of adolescents and adults; type III: spermatocytic seminomas of elderly men; type IV: dermoid cyst; type V: hydatidiform mole.[124] The first three types can arise in the testis from immature germ cells.

Testicular germ cell tumors of adolescents and adults, the seminomas and non-seminomas, belong to the type II tumors and are the most common malignancies in Caucasian men in the second to fourth decade of life.[125] They develop from PGCs/gonocytes that are malignantly transformed and are located in the seminiferous tubules where normally spermatogenesis takes place. These precursor cells of the invasive tumors are known as carcinoma *in situ* (CIS)[126] or intratubular germ cell neoplasia unclassified (ITGCNU)[124] and are normally not found in adult testis. The invasive tumors themselves may exhibit a wide spectrum of histological subtypes, ranging from seminoma to various non-seminomatous elements: EC, teratoma, choriocarcinoma, and yolk sac tumor.[124] Seminoma cells are similar to embryonic germ cells, i.e. PGCs or gonocytes, based on morphology, marker expression profile and differentiation capacity.[123] The malignant counterpart of ES cells, EC cells are in principle pluripotent, and thus able to differentiate into several lineages analogous to normal embryonic development (Figure 2). Besides the testis, type II GCTs can also be found in the ovaries and extragonadal sites, primarily in the midline of the body.[123] A major feature that set these tumors aside from all other solid tumors is their extreme sensitivity to radiotherapy and chemotherapy resulting in a high curability of approximately 95%.[127,128] This response to DNA damage reflects the embryonic origin of these tumors as ES cells are characterized by the absence of G1 arrest after induction of DNA damage.[129] Type II GCTs have a distinct chromosomal constitution with the gain of 12p as the hallmark of all invasive tumors. Gain of chromosome 12, including 12p, is also found upon prolonged culturing of human ES cells suggesting a selective advantage of increased dosage of the short arm of chromosome 12 for the propagation of undifferentiated human ES cells *in vitro* as well as tumor cell proliferation *in vivo*.[130]

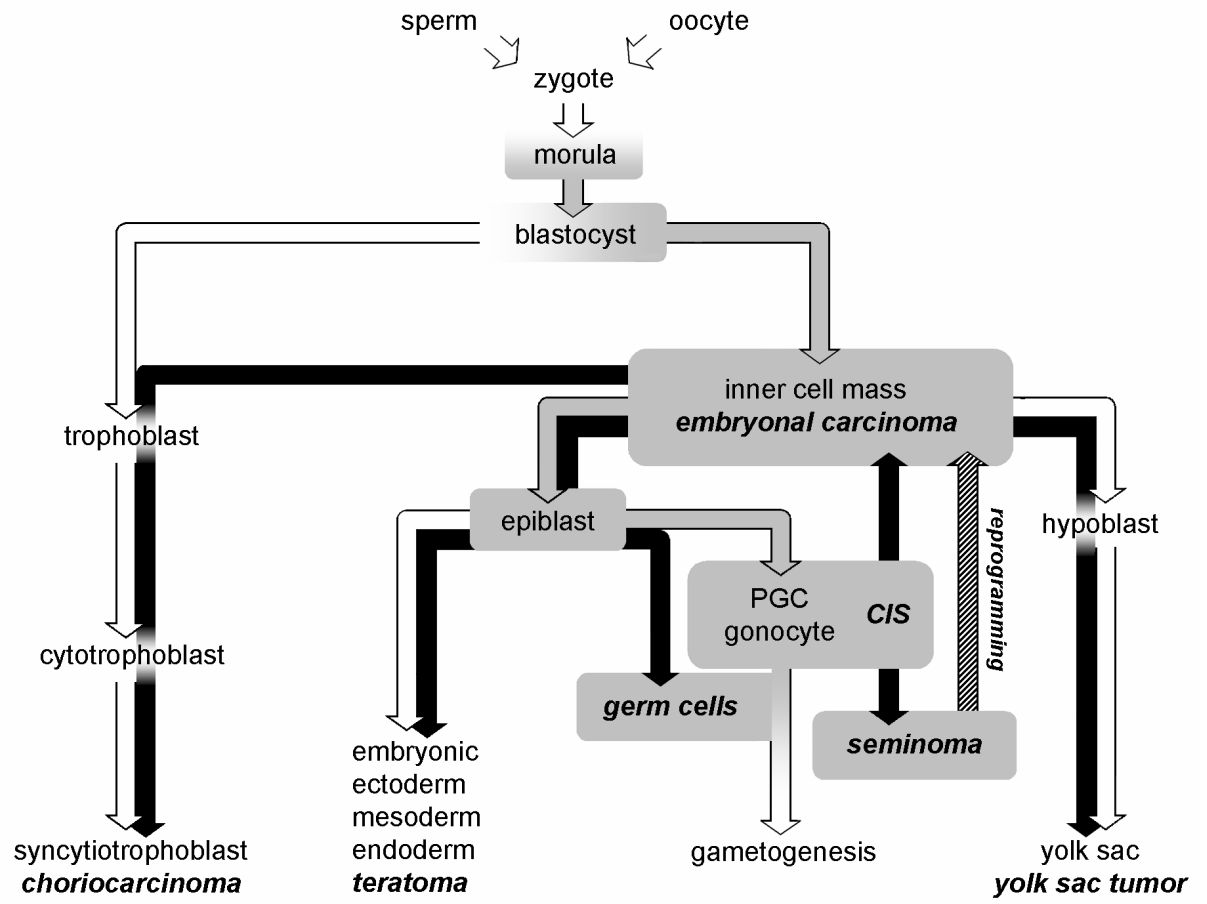

FIGURE 2. OCT3/4 (POU5F1) expression in normal embryogenesis and type II testicular GCT development. Developmental stages and cell types that are OCT3/4 positive are represented in grey. The developmental stages and cell types of GCTs are in bold/italic and grouped with their embryonic counterpart. Starting at the precursor lesion carcinoma *in situ* (CIS), black arrows represent the development into the various extra-embryonic and embryonic histological elements. Seminoma has a limited differentiation capacity and can only form trophoblastic giant cells (not depicted in this scheme), however seminoma cells can be reprogrammed to embryonal carcinoma cells. Embryonal carcinoma is pluripotent and can give rise to all three germ layers. In addition, embryonal carcinoma can give rise to malignant germ cells that have a more mature phenotype than gonocytes as these germ cells also express TSPY.

In contrast to somatic cells, murine ES cells show predominantly loss and reduplication during *in vitro* culturing leading to uniparental disomy.[131] In human ES cells uniparental disomy has not been reported so far, but the repeatedly reported chromosomal instability leading to predominantly gain of sequences favours the establishment of uniparental disomy.[132] Interestingly, uniparental disomy is also found in type II GCTs.[133] Although its presence was explained by a possible fusion of an early germ cell and a sperm, recent own unpublished data indicate that this model cannot be true, because uniparental disomy is also found in ovarian type II GCTs, which can never be the result of sperm fusion.
Expression of OCT3/4 in testicular GCTs on mRNA level was reported in 2002 by Palumbo *et al* [134] who showed expression in seminoma and EC and absence in differentiated elements teratoma and yolk sac tumor. PCR-amplified sequences were examined by restriction enzyme digestion and were specific for the *OCT3/4* gene transcript and not for the *OCT3/4* pseudogenes. The value of OCT3/4 as an

immunohistochemical marker in clinical pathology has been recognized since 2003 when Looijenga *et al* [45] published an extensive study on OCT3/4 protein expression in a multi-tumor tissue microarray analysis covering over 100 different tumor categories and 3600 individual cancers. They verified that the OCT3/4 protein is specific for particular cell types of germ cell tumors. In the testis, the protein was only present in CIS, seminoma and EC, and absent in differentiated tumor elements as teratoma, yolk sac tumor and choriocarcinoma. In addition, expression was absent in normal adult testis including the germ cell lineage. In the ovary, OCT3/4 expression was confined to dysgerminomas, which are the seminomatous equivalent of the female gonad as shown by expression profiling.[135] The precursor lesion of dysgerminoma in the dysgenetic gonad, i.e. gonadoblastoma, was also found to be positive for OCT3/4 in accordance to its testicular equivalent CIS. Finally, four germ cell tumors in the midline of the brain showed positive staining of the EC and germinoma (seminoma of the brain) cells. Comparison of chemosensitive and chemoresistant germ cell tumors showed no difference in staining pattern.[45]

SCOPE OF THIS THESIS

The studies presented in this thesis focus on the role of OCT3/4 in germ cell tumorigenesis and its value as a diagnostic marker for germ cell tumors in clinical pathology. An immunohistochemical marker applied for diagnostic purposes has to fulfill a number of criteria. First, it has to be reliable, reproducible and robust. Second, the expression pattern in normal tissues, both adult and during development, must be precisely determined in order to correctly interpret immunohistochemical results. Therefore, the expression pattern of different OCT3/4 antibodies was investigated in a consecutive series of diagnostic samples. Special attention was given to diagnostic challenging cases in which OCT3/4 is mandatory to come to the final diagnosis. Next to patient samples, OCT3/4 is used to establish the nature of cell lines as proper *in vitro* model systems for germ cell tumors.
The parallels between normal embryonic development and germ cell tumor development are used to come to a deeper insight into the origin and progression of these tumors. The central role of OCT3/4 in maintaining pluripotency and restricting differentiation serves as a starting point for investigation. Epigenetic modification of the promotor region of *OCT3/4* is investigated in the context of expression regulation. Finally, the shift in function from gatekeeper of pluripotency in stem cells to survival factor in germ cells is linked to a cell specific protein partner.

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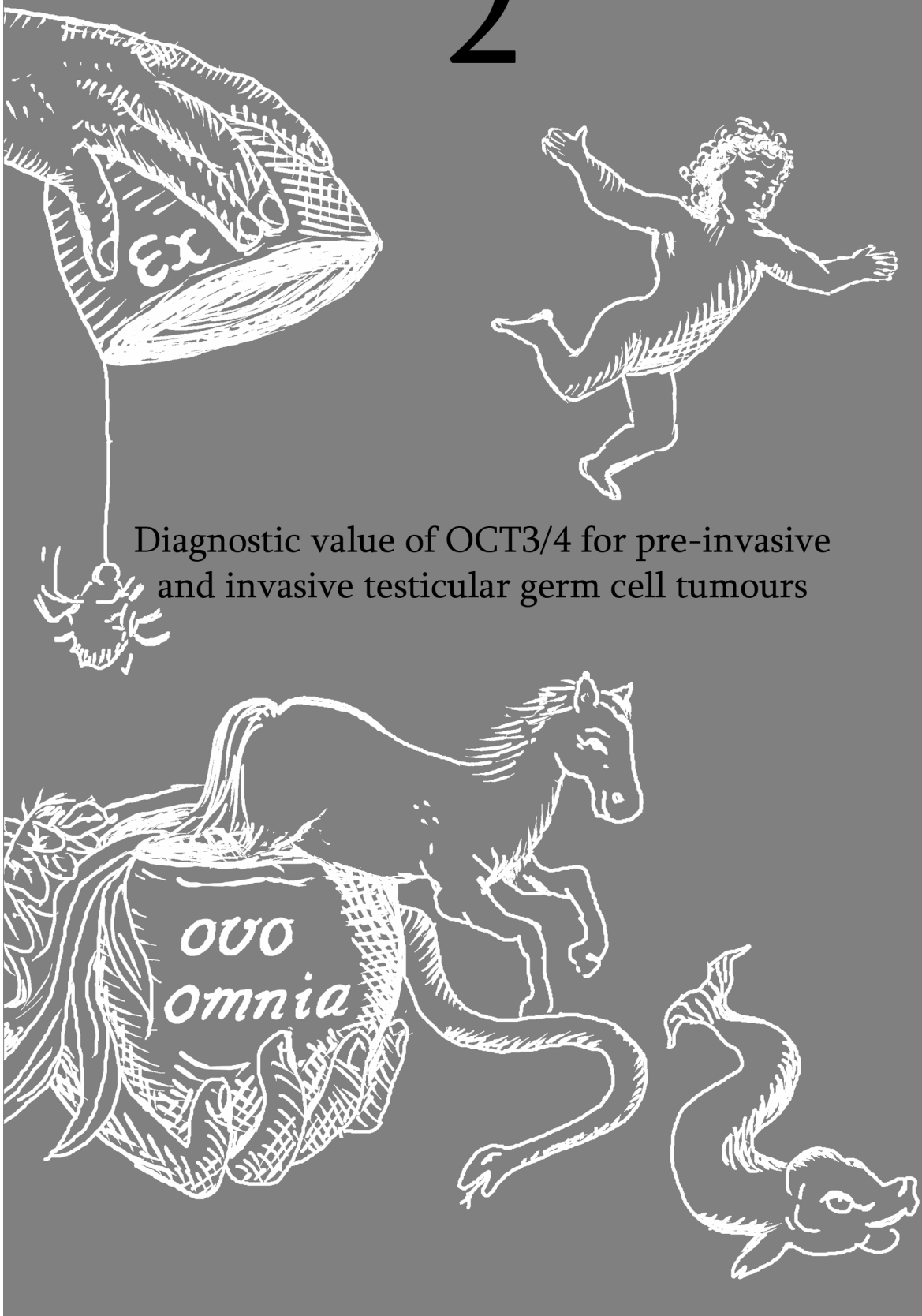

Diagnostic value of OCT3/4 for pre-invasive and invasive testicular germ cell tumours

Diagnostic value of OCT3/4 for pre-invasive and invasive testicular germ cell tumours

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ABSTRACT

Human testicular germ cell tumours of adolescents and adults (TGCTs), the seminomatous and non-seminomatous germ cell tumours, show morphological and biological similarities to normal embryonic development, presumably determined by their supposed cell of origin, the primordial germ cell/gonocyte. Based on this knowledge, OCT3/4, also known as POU5F1, was recently defined as a diagnostic marker for these tumour types. In the adult testis, positive immunohistochemistry for OCT3/4 is an absolute indicator for the presence of the TGCT precursor carcinoma *in situ*/intratubular germ cell neoplasia undifferentiated (CIS/ITGCNU), seminoma, and/or embryonal carcinoma. Several studies have confirmed this observation, using the same polyclonal antibody. The present study demonstrates the usefulness of OCT3/4 immunohistochemistry in a diagnostic setting of a consecutively collected series of more than 200 testicular tumours and over 80 testicular biopsies.

Moreover, it is shown that a monoclonal antibody directed against OCT3/4 is as informative as the polyclonal antibody, both in immunohistochemistry and in western blot analysis. The antibodies are robust and applicable with different methods of pretreatment and storage of tissue. This allows routine application of this diagnostic marker.

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INTRODUCTION

Testicular germ cell tumours of adolescents and adults (TGCTs) - seminomas and non-seminomas - are the most common malignancies in Caucasian men in the second to fourth decades of life [1]. These tumours can be treated effectively using surgery, irradiation, and chemotherapy, resulting in 5- and 10-year survivals in the range of 90–95%. Despite this good prognosis, a major concern is the annual increase in incidence of 2–5% [2].
Invasive TGCTs exhibit a wide spectrum of histological types, ranging from seminoma to various non-seminomatous elements: embryonal carcinoma, teratoma, choriocarcinoma, and yolk sac tumour [3]. Seminoma cells are similar to embryonic germ cells, most likely primordial germ cells (PGCs) or gonocytes. Embryonal carcinoma is the malignant counterpart of embryonic stem cells, in principle pluripotent, and thus able to differentiate into all three germ lineages. This histological spectrum mimics embryonal development to a certain extent (see ref 4 for a review).
Despite the palette of histological appearances of TGCTs, they all originate from a common precursor, known as carcinoma *in situ* (CIS) [5] or intratubular germ cell neoplasia unclassified (ITGCNU). Recent findings regarding the expression of embryonic traits, and the high expression of the cell cycle regulators that are typical of embryonic mitotic germ cells in CIS cells, support the hypothesis that these cells originate from PGCs/gonocytes (see ref 6 for a review). So far, all known markers of CIS cells are also found in immature germ cells [7]. This hampers the applicability of these markers at an early age, particularly if a delay or block of maturation of germ cells is expected, for example in the case of cryptorchidism or gonadal dysgenesis [8]. The presence of immature germ cells may result in overdiagnosis of CIS, and therefore possibly overtreatment.
To detect CIS by means of immunohistochemistry, the markers c-KIT [9] and PLAP [10] have been used for more than a decade. One of the most recent markers is OCT3/4, also known as POU5F1. This transcriptional regulator is expressed in undifferentiated, pluri-potent cells including embryonic stem cells and germ cells, both mouse [11–13] and human [14,15]. Knock-out mice are non-viable, because they fail to form a pluripotent inner cell mass, but instead consist primarily of cells committed to the trophoblast lineage [16]. Inhibition of expression of this gene in mouse and human embryonic stem cells results in a similar type of differentiation induction to trophectoderm [17,18]. In contrast, recent data show that PGCs without OCT3/4 do not differentiate, but undergo apoptosis [19]. This indicates that the role of OCT3/4 in embryonic stem cells and PGCs is different.
We showed that expression of OCT3/4 in TGCTs is highly similar to the pattern found during normal development. In fact, testicular parenchyma containing CIS, as well as seminoma and embryonal carcinoma cells, contained mRNA of this gene, while it was absent in differentiated non-seminomas [20]. Subsequently, we demonstrated, by using a polyclonal antibody, that OCT3/4 protein is detectable in the cell types also positive for the mRNAs [21]. We applied immunohistochemistry to a large series of solid tumours (organized in tissue microarrays, TMAs) and found that OCT3/4 is a specific marker for CIS, seminoma, and embryonal carcinoma. In addition, its expression was also found in gonadoblastoma (the CIS counterpart of dysgenetic gonads), dysgerminoma, and

germinoma (the seminomatous tumours of the ovary and midline of the brain, respectively). No correlation between treatment response and OCT3/4 staining was observed. None of the non-germ cell tumours investigated expressed the protein, except for three isolated cases [21]. Subsequently, these findings were confirmed by different studies [8,22–27] (see Table 1). In addition, Baker and Oliva recently reviewed the value of diagnostic markers in ovarian tumours, in which again OCT3/4 was concluded to be suitable for identifying dysgerminomas and ovarian embryonal carcinomas [28].

Here we report on our diagnostic experience of using immunohistochemistry for OCT3/4 on a consecutive series of more than 200 testicular cancers. Moreover, the applicability for the diagnosis of CIS in testicular biopsies is shown. We demonstrate that, in addition to the polyclonal antibody, which has been used in various studies, a suitable monoclonal antibody is now available that is informative for both immunohistochemistry and western blotting. The monoclonal antibody assures infinite availability of OCT3/4 for clinical pathology and research. The diagnostic value of OCT3/4 immunohistochemistry is exemplified by the presentation of two patients. One presented with an unusual tumour in the brain and the other with a highly necrotic tumour in the retroperitoneal region. The positive staining of the neoplasms for OCT3/4 led to their recognition as germ cell tumours and the patients were treated successfully.

TABLE 1. Chronological summary of published data on OCT3/4 in germ cell tumours at the mRNA and protein levels

		Germ cell tumours													
		Pre-invasive			Invasive									Metastatic	
					Seminomatous				Nonseminomatous						
Reference	Method	SpA	CIS	GB	SE	GE	DG	SpSE	EC	YST	MTE	ITE	CHC	EC	SE
Looijenga et al. [21]	IHC	-	+	+	+	+	+	-	+	-	-	-	-		
Gidekel et al. [24]	IHC	+	+		+				+						
Sperger et al. [22]	Microarray				+				+	-					
Rajpert-De Meyts [8]	IHC	-	+	+	+			-	+						
Jones et al. [25]	IHC				+			-	+	-	-	-	-		
Almstrup et al. [23]	Microarray		+		+				+						
Cheng et al. [26]	IHC			+			+			-	-	-			
Cheng et al. [34]	IHC													+	+
Jones et al. [27]	IHC		+												

All immunohistochemical studies were performed with a polyclonal goat anti-human OCT3/4 antibody (C20, sc 8629; Santa Cruz Biotechnology, Santa Cruz, CA, USA), except for Gidekel *et al*, where a self-made rabbit anti-mouse OCT3/4 antibody was used.

IHC = immunohistochemistry; SpA = spermatogonium A; CIS = carcinoma *in situ*; GB = gonadoblastoma; SE = seminoma; GE = germinoma; DG = dysgerminoma; SpSE = spermatocytic seminoma; EC = embryonal carcinoma; YST = yolk sac tumour; MTE = mature teratoma; ITE = immature teratoma; CHC = choriocarcinoma.

MATERIALS AND METHODS

Sample handling and characterization

Use of tissues for scientific purposes was approved by the institutional review board (MEC 02.981). The samples were used according to the 'Code for proper secondary use of human

tissue in The Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).
A total of 223 testicular tumours were consecutively collected in the south-western part of The Netherlands, in collaboration with urologists and pathologists. Representative parts of the tumour (and adjacent tissue, if available) were snap-frozen in liquid nitrogen or fixed in 10% formalin overnight for paraffin wax embedding. Tumours were diagnosed according to the WHO classification, supported by immunohistochemistry using antibodies directed against germ cell/placental alkaline phosphatase (PLAP), α-fetoprotein (AFP), human chorionic gonadotropin (hCG), the stem cell factor receptor c-KIT, and cytokeratin 8 (CAM5.2). CD30 was used on a selected series. In addition, 81 open surgical biopsies from 56 adults with primary infertility or a history of TGCT were evaluated for Johnson score and the presence of CIS.

Immunohistochemistry

Immunohistochemistry with anti-OCT3/4 antibodies was performed on paraffin wax-embedded tissue sections of 3 μm thickness. Endogenous peroxidase activity was inactivated by incubation in 3% H2O2 for 5 min. Antigen retrieval was carried out by heating sections in 0.01 M sodium citrate (pH 6.0) under high pressure up to 1.2 bar. Endogenous biotin was blocked using an avidin/biotin blocking kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). The sections were incubated for 2 h at room temperature with a polyclonal goat anti-human OCT3/4 antibody (sc-8629; Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against the COOH terminus of the protein. This antibody is used in almost all studies of OCT3/4 and germ cell tumours (Table 1). The antibody was diluted 1 : 1000. Subsequently, a biotinylated horse anti-goat secondary antibody (BA-9500; Vector) diluted 1 : 200 was applied and bound antibody complex was visualized using the horseradish peroxidase (HRP) avidin–biotin complex method. Sections were counterstained with haematoxylin.
A monoclonal antibody against the N-terminus of human OCT3/4 (sc-5279; Santa Cruz Biotechnology) was compared with the polyclonal antibody using a 1 : 1000 dilution in the same assay as described above. As a secondary antibody, a biotinylated rabbit antimouse antibody was used (E0413; Dako, Glostrup, Denmark). Detection and counterstaining were as described above.
To compare the staining pattern of the monoclonal and polyclonal anti-OCT3/4 antibodies, immunohistochemistry was performed on parallel paraffin wax sections. In addition, both antibodies were used at a dilution of 1 : 4000 on parallel, 4 μm thick and acetone-fixed, frozen tissue sections.
Initial OCT3/4 staining of the brain tumour from patient 2 was performed on limited paraffin waxembedded material that was left after a large panel of markers was tested. Therefore, only a small fragment containing tumour cells was present. To improve this result, a negative control slide was reused for OCT3/4 immunohistochemistry using the monoclonal antibody.

Immunofluorescence

Fluorescent immunohistochemistry was performed on acetone-fixed frozen tissue sections of 4 μm thickness and cytospins of cell lines. The monoclonal anti-OCT3/4 antibody was incubated 1 : 2000 for 1 h at room temperature. Subsequently, a secondary FITClabelled goat anti-mouse antibody (1090-02; Southern Biotechnology Associates, Birmingham, AL, USA) was incubated for 30 min. Counterstaining was done with Vectashield (Vector)/ DAPI (Sigma Chemical Co, St Louis, MO, USA).

Cell culture

The human cell lines Tera1 [29], 2102Ep [30], NT2 [31], and NCCIT [32], all of which were derived from embryonal carcinomas, were grown in a 5% humidified incubator until 80% confluency was reached. Cells were incubated with trypsin–EDTA, harvested, and cytospins were prepared.

Protein isolation

Tissue from frozen sections containing normal testis, embryonal carcinoma, yolk sac tumour, mature teratoma and seminoma, and pellets from cell lines Tera1, 2102Ep, NT2, and NCCIT were used to extract protein. Samples were washed in cold PBS and centrifuged at 0 ◦C for 5 min at 1500 rpm. Lysis buffer (1× PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS) including protease inhibitors (1 836 153 complete mini cocktail; Roche Diagnostics, Mannheim, Germany) was added. Samples were sonicated and centrifuged at 0 ◦C for 10 min at 14 000 rpm. Protein lysates were stored at - 80 ◦C.

Western blot

Samples of 20 μg protein were run on a 10% SDSpolyacrylamide gel, blotted on a PVDF membrane for 1 h, and incubated with a primary antibody overnight at 4 ◦C (anti-Oct3/4 monoclonal and polyclonal antibody both diluted 1 : 1000). HRP-labelled secondary antibody was incubated for 1 h at room temperature. After washing, ECL western blotting detection reagents (RPN2109; Amersham Biosciences, UK) were added for 5 min and the blots exposed. After scanning, the blots were stripped and re-probed with anti-β-actin (1 : 5000) as a loading control.

RESULTS

Immunohistochemistry

To test the diagnostic use of OCT3/4, immunohistochemistry was performed using the polyclonal antibody on a series of consecutively collected cases. In total, 209 testicular germ cell tumours were investigated after radical orchidectomy: three spermatocytic seminomas and 206 TGCTs (110 pure seminomas, 50 pure non-seminomas, and 46 combined tumours, the latter containing both a seminoma and a non-seminoma component). Mean age and range in major tumour groups were according to the

literature. None of the three spermatocytic seminomas or 14 non-germ cell tumours [five B-cell lymphomas, three Leydig cell tumours, three embryonal rhabdomyosarcomas, one sarcoma, one carcinoid, and one metastatic renal carcinoma (clear cell type)] stained for OCT3/4. In contrast, all seminoma and embryonal carcinoma components were positive. CIS cells in the adjacent parenchyma of seminomas, non-seminomas, and combined tumours were all consistently positive. This was also true for intratubular seminoma (n = 29) and embryonal carcinoma (n = 18). There was a sharp transition between positive and negative cells, coinciding with the border of CIS, seminoma, and embryonal carcinoma with adjacent tissue. The CIS and seminoma cells showed highly consistent and strong nuclear staining, whereas in embryonal carcinoma cells, diffuse cytoplasmic staining was seen in addition to the nuclear staining. Representative examples are shown in Figures 1A and 1B (seminoma in Figure 1A; seminoma and embryonal carcinoma in Figure 1B). Fluorescent detection confirmed this cytoplasmic staining to be absent in seminoma (Figure 1C) and CIS, and specific for embryonal carcinoma (Figure 1D). Because cytoplasmic staining was never seen in the negative controls, and was only found together with nuclear presence of the protein, we regard cytoplasmic staining to be an intrinsic characteristic of embryonal carcinoma. Based on morphological and immunohisto-chemical data, no CIS, seminoma or embryonal carcinoma cell was found to be negative. This pattern of staining is in agreement with our previous observations, in line with the published data of others (Table 1).

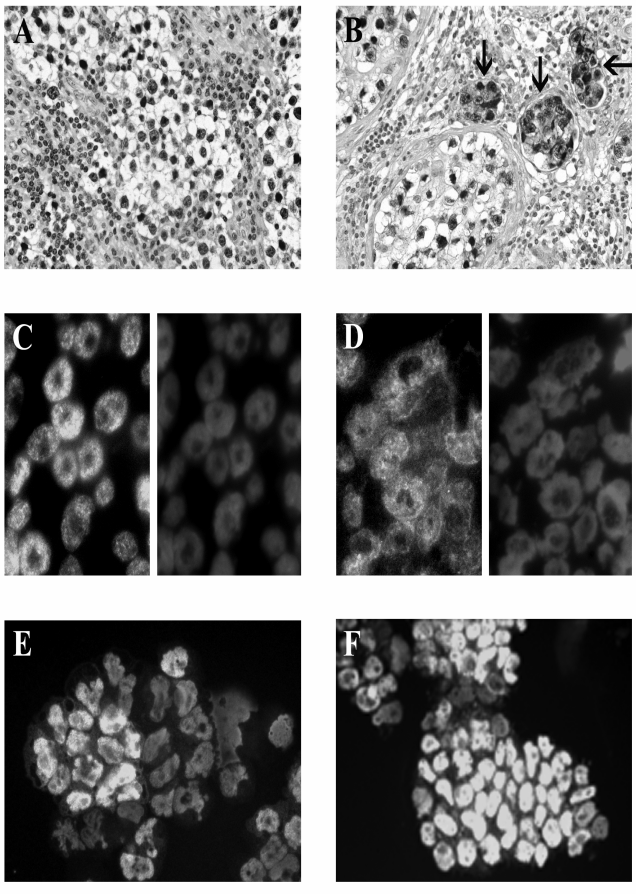

FIGURE 1. OCT3/4 immunohistochemistry using different embedding, fixation, and detection methods. (A) Nuclear staining (brown) of seminoma cells with a mono-clonal antibody. (B) Embryonal carcinoma and seminoma incubated with a polyclonal antibody. Note the cytoplasmic staining in the angio-invasive embryonal carcinoma (arrows) compared with the clear cytoplasm of intratubular seminoma cells. Both slides are from formalin-fixed and paraffin wax-embedded tissue. Images C–F show FITC-labelled fluorescence with the monoclonal antibody as the primary step. Nuclear staining is in blue (DAPI) and OCT3/4 localization in green (FITC). (C) Frozen tissue from a seminoma. (D) Frozen tissue from an embryonal carcinoma. Note the difference in cytoplasmic staining pattern between seminoma and embryonal carcinoma cells. Cytospin preparations of (E) pluripotent embryonal carcinoma-derived cell line NT2 and (F) nullipotent 2102Ep. Note the OCT3/4-negative cells located at the periphery of the aggregates.
(Figure 1 colour image on page 178)

Besides the different tumour samples, four embryonal carcinoma-derived cell lines were investigated. All cell lines showed strong nuclear and faint cytoplasmic staining with both enzyme-based and fluorescent immunohistochemistry. Remarkably, all cell lines contained a number of cells without positive nuclear OCT3/4 staining. This heterogeneity was clearly greater in the pluripotent NT2 cell line (41% OCT3/4-negative) than in nullipotent cell lines [2102Ep (9%), NCCIT (18%), and Tera1 (14%)], possibly due to spontaneous differentiation (Figures 1E and 1F). OCT3/4-negative cells were predominantly located at the borders of cell aggregates, which is consistent with differentiation [33].

Next to the analysis of orchidectomy specimens (see above), a series of consecutively collected testicular biopsies was included. These were taken because of infertility or a history of TGCT. In total, 81 biopsies from 56 patients were included. Based on both morphology and immunohistochemistry for OCT3/4, CIS was found in open surgical biopsies in four patients: two had a prior TGCT of the contralateral testis and two presented with infertility. A clinically occult seminoma was diagnosed in the biopsy from another patient with prior TGCT. In addition, in two patients with a palpable testicular node, the excision biopsy showed seminoma in both cases.

Comparison of the monoclonal and polyclonal antibodies

Because of the, by definition, finite availability of a polyclonal antibody, and possible differences between batches, we studied the suitability of a commercially available monoclonal antibody directed against OCT3/4. In parallel, this monoclonal and the polyclonal antibody used to date (also see above) were compared using immunohistochemistry on both frozen and formalin-fixed, paraffin wax-embedded tissue, and western blot analysis. Both antibodies showed a similar pattern in both approaches, without background staining (data not shown). Western blot analysis was performed on total protein extracted from normal testis, seminoma, and the various histological types of non-seminoma, as well as the TGCT-derived cell lines (Figure 2A). These results are in accordance with the immunohistochemical data. The presence of the two bands around the expected size of 43 kD, corresponding to the longer OCT3/4 variant [13], is currently under investigation.

Robustness of the polyclonal and monoclonal antibodies for immunohistochemistry

To check the stability of the polyclonal and monoclonal antibodies for immunohistochemistry, the following procedure was performed repeatedly (n = 4). Undiluted antibody was frozen at –20 ◦C, thawed, used for immunohistochemistry, and again stored at –20 ◦C. Staining results did not decrease after multiple cycles. Immunohistochemistry results for frozen acetone-fixed, paraffin wax-embedded, formalin-, formaldehyde- and Bouin (Figure 2B)-fixed material and pretreated by microwave or high pressure were all successful, and suitable for diagnostic purposes.

Demonstration of the clinical value of OCT3/4 immunohistochemistry

The clinical value of immunohistochemistry for OCT3/4 in the diagnosis of solid tumours is demonstrated by the following two cases.

Case 1

A 37-year-old male presented with pain in the left flank for 1 year, related to an abdominal mass extending from the diaphragm to the aortic bifurcation with involvement of the left kidney and retroperitoneal lymphadenopathy. The patient showed small testicles on ultrasound. Serum LDH was strongly elevated up to 3568 U/l (normal: 0–449), while hCG levels were elevated to 29.5 IU/l (normal: 0–1.9). Guided biopsies showed large areas of necrosis and fibrosis with fields and strands of closely packed neoplastic cells with intermingling of lymphocytes. The malignant cells had round nuclei with prominent nucleoli and intermediate to large amounts of clear cytoplasm. The morphological differential diagnosis was: seminoma, Ewing/small blue round cell tumour and lymphoma. Immunohistochemically, the tumour cells showed very weak cytoplasmic and focally membranous staining for c-KIT (Figure 2C), while scattered cells were positive for keratin 8 (not shown). No staining was found in tumour cells for PLAP, hCG, CD30, AFP, CD99, desmin, CD21, CD45, EMA, and TDT. The lymphocytes were positive for CD45. The tumour cells were consistently positive for OCT3/4 (Figure 2D), from which the diagnosis of seminoma was made and the patient was accordingly treated with chemotherapy. During standard chemotherapy, there was a rise in AFP levels, unusual for a seminoma. A nonseminomatous component was therefore suspected and the patient underwent retroperitoneal lymph node dissection (RPLND) and nephrectomy of the involved left kidney. In addition, his left testis, which showed microlithiasis on ultrasound, was removed. AFP levels normalized and no viable tumour tissue was found in the RPLND, kidney or testis specimens. After a rise in hCG level to 5.8 IU/l suggesting residual tumour, his right testis was removed; again, no tumour tissue was found and hCG normalized. Two and a half years after the initial diagnosis, the patient has no signs of recurrent disease.

Case 2

A 40-year-old female presented with visual disturbances, followed by slowly progressive left-sided motor and sensory disturbances. She also suffered from memory disturbances and was mildly spatially and temporally disorientated. A T2 weighted MRI scan showed a large area of high signal intensity in the periventricular white matter of the right more than the left occipital region and the corpus callosum, without enhancement on T1 weighted images after gadolinium administration and without mass effect. A tumour was considered unlikely and an acute demyelinating encephalomyelopathy was suspected. Repeated cerebrospinal fluid examination showed mononuclear pleiocytosis (10 cells per cubic mm; normal value: <3 cells), without tumour cells. Flow cytometry showed predominantly reactive T-lymphocytes, without evidence of tumour cells. A CT scan of the thorax and abdomen did not reveal any abnormalities. The CSF-serum IgG index was normal and oligoclonal bands were absent. No evidence of vasculitis or an inflammatory

disease was found, and treatment with steroids was ineffective. A stereotactic biopsy of the intracerebral lesion showed blast-like neoplastic cells within a mononuclear infiltrate (Figure 2E). A wide panel of markers including those for a primary central nervous system lymphoma was applied: CD3, CD4, CD5, ALK-1, CD19, CD20, CD79a, CD45, CD30, S100, MELAN-A, HB45, CD68, CD43, PLAP, hCG, AFP, and CD56 all showed no staining; there was some punctate keratin positivity and no final diagnosis was made. At first, her condition stabilized, but after a few months it deteriorated further and an MRI scan showed increasing focal areas of enhancement. Immunohistochemistry for OCT3/4 (Figure 2F) revealed the tumour to be a germinoma and she was treated with whole brain irradiation. Follow-up MR imaging showed disappearance of the enhancement and her condition improved, although focal deficits remained present. One and a half year after treatment, her condition is stable.

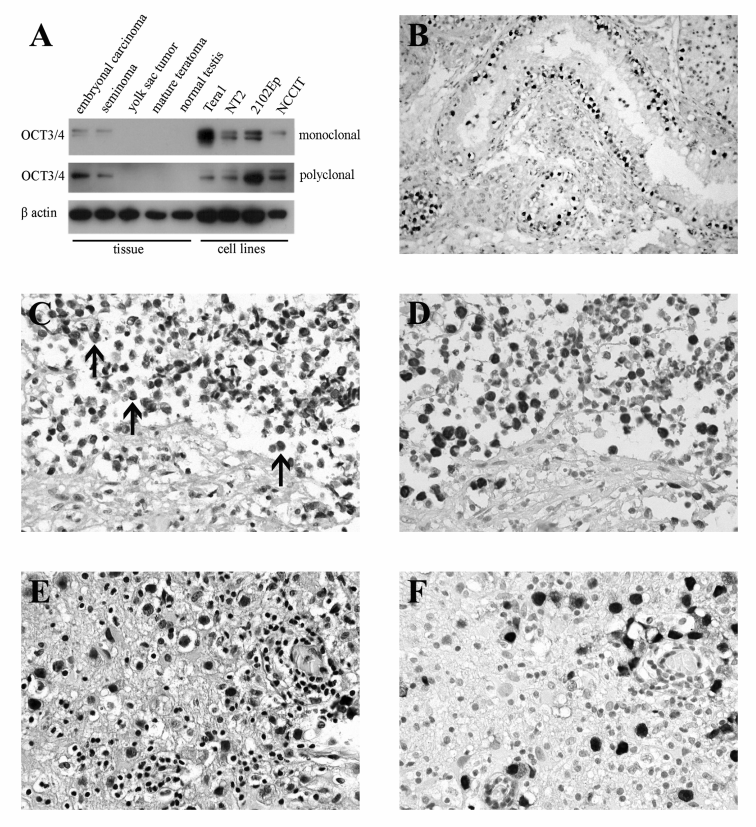

FIGURE 2. (A) Western blot analysis for OCT3/4 in different testicular germ cell tumours, derived cell lines, and normal testis. Note the specificity for seminoma and embryonal carcinoma. (B) OCT3/4 immunohistochemistry on Bouin-fixed tissue showing positive staining of CIS. (C, D) Retroperitoneal tumour with extensive necrosis from patient 1 showing weak cytoplasmic, focally membranous staining for c-KIT in red (C, positive cells indicated with arrows) and distinct brown nuclear staining for OCT3/4 (D). (E, F) Tumour localized in the brain of patient 2, with scattered cells with clear cytoplasm and prominent nucleoli (E) with positive staining for OCT3/4 (F). *(Figure 2 colour image on page 179)*

DISCUSSION

After our initial publication [21] showing the sensitivity and specificity of OCT3/4 immunohistochemistry for the detection of a number of specific histological variants of human germ cell tumours, the results were largely confirmed in subsequent studies, both by immunohistochemistry with the same polyclonal antibody and at the mRNA level (Table 1). The first study included an extensive multi-tumour tissue microarray covering more than 100 different tumour categories and 3600 individual cancers. Strong nuclear expression was found in seminomatous tumours, irrespective of anatomical localization

(like germinoma and dysgerminoma), and embryonal carcinoma, and their common precursor cell CIS (as well as gonadoblastoma), whereas the differentiated components of nonseminomas (yolk sac tumour, teratoma, and choriocarcinoma) were consistently negative. No correlation with chemotherapy sensitivity or resistance was found. Since then, it has been demonstrated that OCT3/4 immunohistochemistry is of value to diagnose metastatic seminoma and embryonal carcinoma [34]. The antibody applied in the different studies reported so far is of polyclonal origin, generated in goat. Therefore, availability of this antibody is finite. Here we have demonstrated for the first time that similar results can be obtained using a mouse monoclonal antibody, the availability of which is unlimited. These antibodies give the same results in immunohistochemistry and western blot analysis. Both antibodies appear to be robust and highly stable, and the immunohistochemical detection method is relatively insensitive to pretreatment and poor quality of the tissue samples. That OCT3/4 is indeed of additional value in clinical pathology compared with the other available markers is demonstrated by the two cases reported in this paper. One of these patients had an unusual presentation of a germinoma (seminoma of the brain) and the other patient had a highly necrotic retroperitoneal seminoma, and both were successfully treated as such. OCT3/4 was the only positive marker found, even when applied on a slide used previously as a negative control. The negativity of the other markers could be due to the absence of these markers in the tissue under investigation and/or poor quality of the tissue.

The value of using immunohistochemistry for OCT3/4 to screen for CIS in testicular parenchyma biopsies taken in the context of an outpatient andrology clinic is also presented. The data again demonstrate the usefulness of OCT3/4 immunohistochemistry for the diagnosis of these pre-invasive cancer cells. It must be kept in mind that the presence of OCT3/4-positive cells in testicular parenchyma of patients below the age of 1 year does not justify the diagnosis of CIS [7], in particular when a germ cell maturation delay or block is expected. For this specific application, additional criteria have to be developed. Recently, Tai *et al* [35] reported OCT3/4 protein expression in some cells in the basal layer of human skin, corresponding in their hypothesis to the presence of adult stem cells. To verify these results, we incubated a total of 20 skin sections from five different individuals with the same monoclonal OCT3/4 antibody; no positive cells were found and this discrepancy remains to be resolved.

The data on OCT3/4 are also highly interesting from the biological point of view. An oncogenic driving force of OCT3/4 in the pathogenesis of TGCTs has been suggested based on mouse studies [24]. This conclusion was drawn from results obtained with experiments using mouse embryonic stem cells, which have various expression levels of OCT3/4. These cells give rise to so-called teratocarcinomas, which are not the proper animal model for TGCTs (see ref 36 for a review). In addition, it has been shown that the effect of OCT3/4 on a PGC and an embryonic stem cell is indeed different [19]. The fact that, in TGCTs, OCT3/4 expression mimics the pattern found during normal development (ie no OCT3/4 expression is detected in the differentiated non-seminomatous components) supports the model that OCT3/4 is under the control of physiological expression machinery and is not an oncogene in its classical sense. Our findings and those of others are in line with OCT3/4 expression being influenced by neither ploidy nor parental genome composition

[37]. In fact, TGCTs originate from an erased PGC/gonocyte [38] and are consistently aneuploid [39]. It remains to be established whether epigenetic modifications have a regulatory role in the expression of OCT3/4 in TGCTs, as indicated in mouse cells [40].
In conclusion, we have demonstrated the diagnostic value of OCT3/4 immunohistochemistry for CIS, seminoma, and embryonal carcinoma in a clinical setting. Use of the monoclonal antibody is preferable for this application.

ACKNOWLEDGEMENTS

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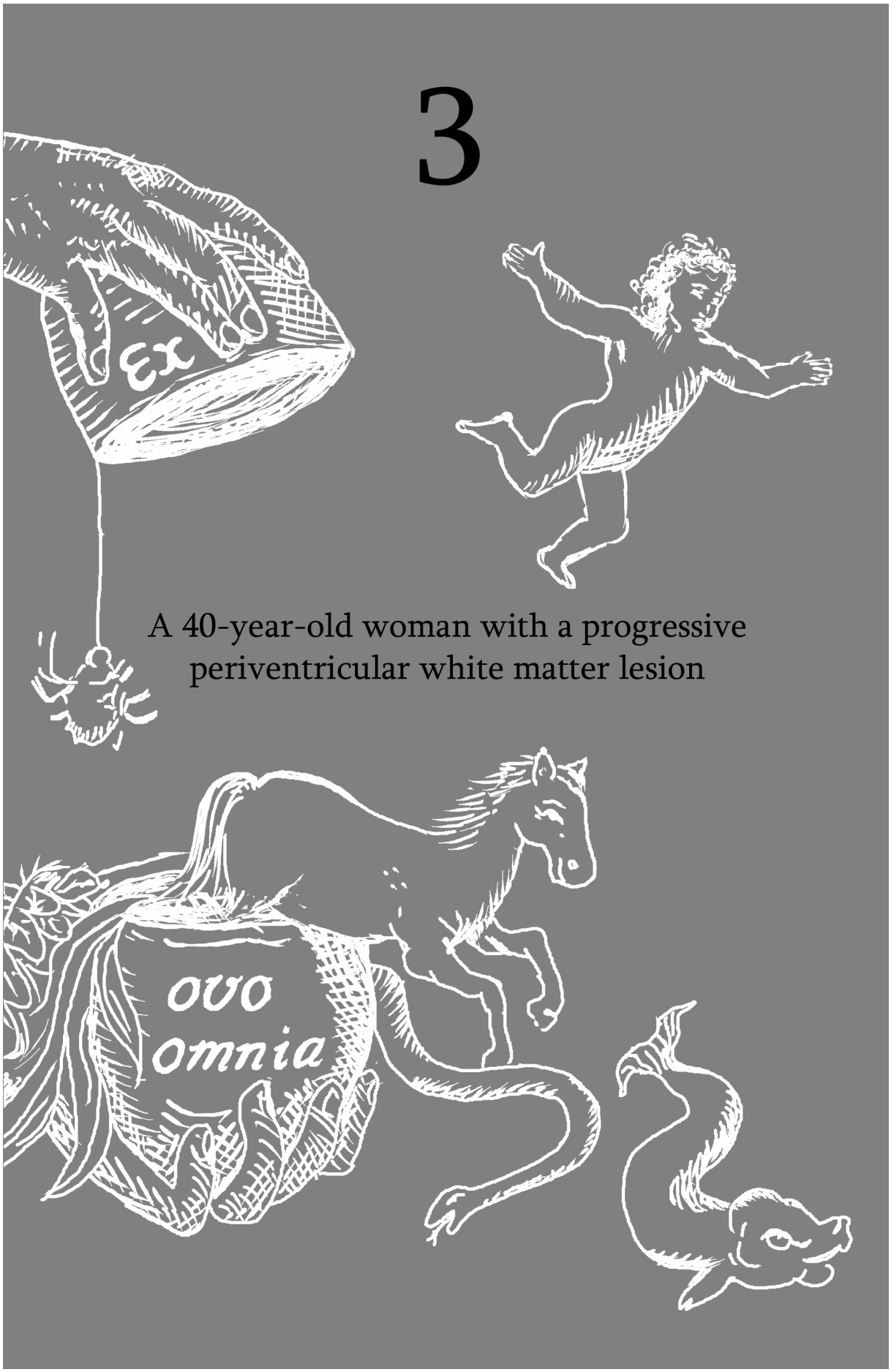

A 40-year-old woman with a progressive periventricular white matter lesion

A 40-year-old woman with a progressive periventricular white matter lesion

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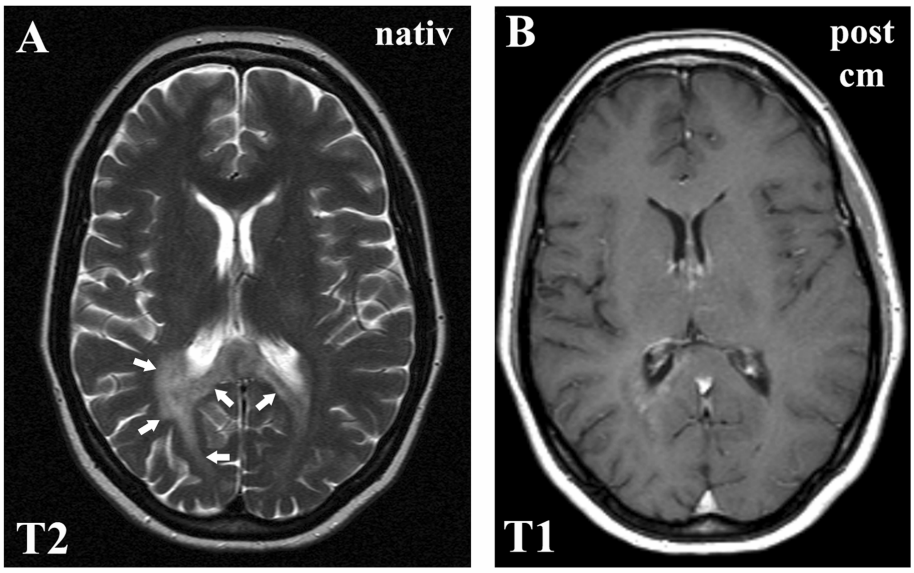
CLINICAL HISTORY

A 40-year-old female presented with blurred vision and diplopia, followed by slowly progressive left-sided motor and sensory disturbances. She also suffered from memory loss and mild spatial and temporal disorientation. A T2 weighted MRI (Figure 1A) showed a large area of high signal intensity (indicated by arrows) in the periventricular white matter of the right more than the left occipital region and the corpus callosum, without enhancement on T1 weighted images (Figure 1B), There was no mass effect. A tumor was considered unlikely, and acute demyelinating encephalomyelopathy (ADEM) was suspected. Repeated cerebrospinal fluid (CSF) examinations showed a mononuclear pleocytosis (10 cells per cubic mm, normal value: <3 cells), without immunohistochemical evidence of tumor cells; tumor markers HCG, AFP and CEA were not elevated. Flow cytometry showed predominantly reactive T-lymphocytes, but again no evidence of tumor cells. CT scan of thorax and abdomen did not reveal any abnormalities. The CSF-serum IgG index was normal, and oligoclonal bands were absent. No evidence of a vasculitis or an inflammatory disease was found. Treatment with steroids proved ineffective.



Brain Pathology Case of the Month - August 2007
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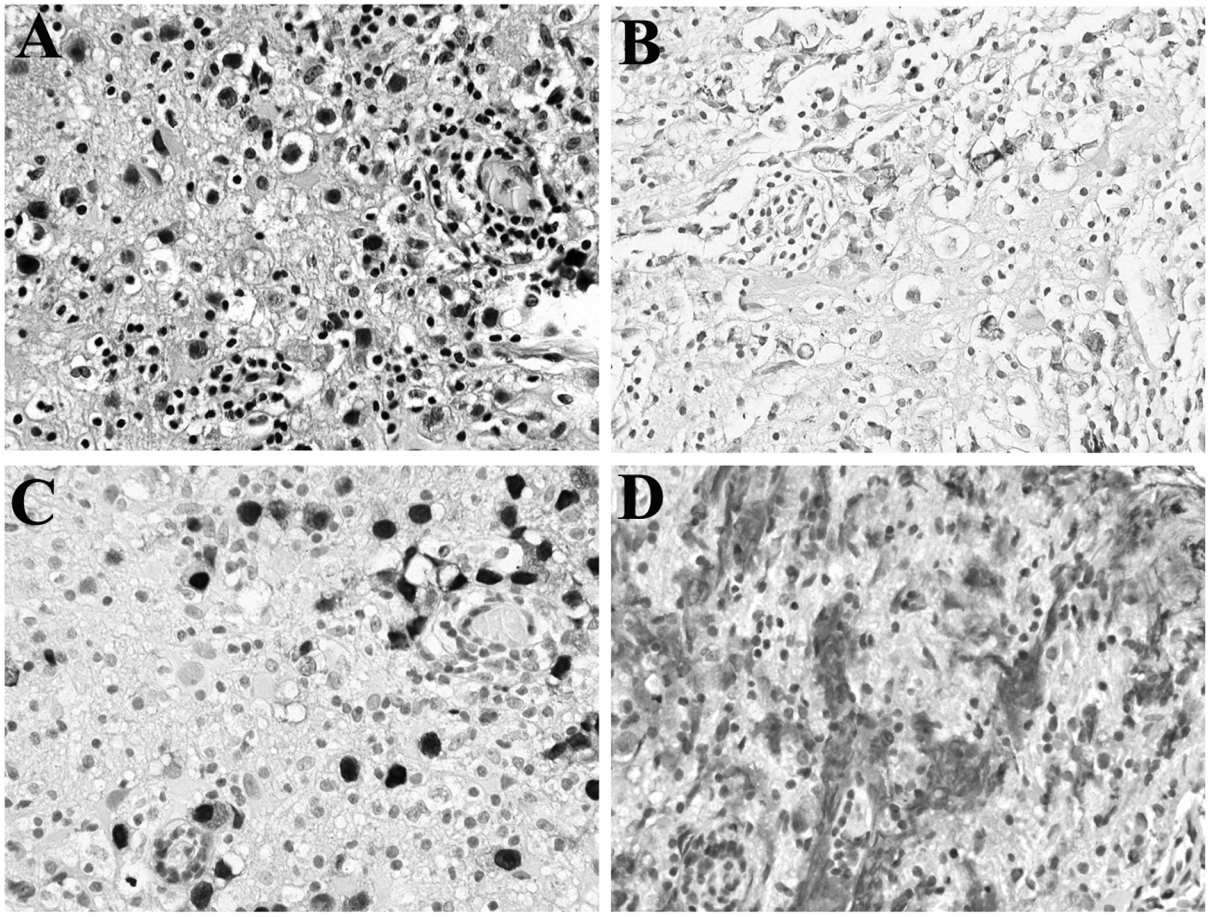
MICROSCOPIC DESCRIPTION

A stereotactic biopsy of the intracerebral lesion showed blast-like neoplastic cells within a mononuclear infiltrate (Figure 2A). The rounded tumor cells contained a large centrally located nuclei. A wide panel of markers including those for carcinoma, melanoma and primary central nervous system lymphoma was applied: CD3, CD4, CD5, ALK-1, CD19, CD20, CD79a, CD45, CD30, S-100, MELAN-A, HMB45, CD68, CD43, PLAP, hCG, AFP and CD56, all found to be negative; there was some punctuated NCL5D3 (low molecular weight keratins 8 and 18) positivity (Figure 2B). No final diagnosis could be made.



Subsequently, the marker OCT3/4 became available, which has proven to be specific for certain histological types of germ cell tumors (6), including seminomatous tumors and embryonal carcinoma. This has been confirmed in multiple independent studies (1) (for review). 100% of tumor cell nuclei present in the biopsy of the above mentioned patient clearly stained positive for OCT3/4 (Figure 2C). Because only a small number of tumor cells were present in this slide and no biopsy material was left anymore, OCT3/4 was also applied to a slide previously found to be negative by immunohistochemistry for another unrelated marker. Again all tumor nuclei stained positive for OCT3/4 without any background. Recently, the stem cell factor receptor c-KIT was also applied on a previously negative slide and the cytoplasm of some tumor cells was stained positive (Figure 2D).

DIAGNOSIS

Diffusely infiltrating germinoma

ADDITIONAL TREATMENT AND CLINICAL PROGRESS

After the lesion was diagnosed as a germinoma, the patient was treated with whole brain irradiation in fractions of 1.6 Gy, to a total dose of 44.8 Gy. Follow-up MRI showed disappearance of the enhancement and her condition improved, although focal deficits remained present. Three years after treatment she had a relapse which was treated with chemotherapy (bleomycin-etoposide-cisplatin) with good respons. One year later a second relapse occurred

DISCUSSION

Germinomas are tumors of germ cell origin and represent the brain counterpart of testicular seminoma. They are relatively rare and represent 0.4-3.4% of all primary intracranial tumors, although in Northeast Asia this is significantly higher, up to 9% (4). Like other extragonadal germ cell tumors, germinomas occur primarily in the midline, with the region of the pineal gland being the most common site, followed by the suprasellar compartment (8). They occur predominantly in children with a peak incidence around 12 years of age (4).

Depending on anatomical localization, patients with a germinoma may present with a range of neurological, endocrine and psychiatric symptoms. Pineal region tumors often compress the cerebral aqueduct, resulting in progressive hydrocephalus, and invade the tectal plate, producing a paralysis of upward gaze and convergence known as Parinaud syndrome. Suprasellar germ cell tumors compress the optic chiasm, resulting in visual loss, and present with endocrinopathy due to pituitary failure with diabetes insipidus, retarded growth and sexual maturation (8). Cure rates for this tumor exceed 90% at 10 years, and limitation of treatment-related late morbidity is therefore essential (7).

In this case, the characteristic radiological features of a germinoma were absent (3, 5). There was no mass effect, i.e. compression of pre-existent anatomical structures by growth of a solid tumor, and no enhancement after gadolinium administration. Instead, the tumor was diffusely infiltrating the surrounding tissue and followed the contours of the ventricular system. In addition, the marker placental alkaline phosphatase (PLAP) usually present in germinomas (2) was negative and diagnosis was made on nuclear OCT3/4 staining, which has proven to be a valuable marker in diagnosis of germ cell tumors (6, 1).

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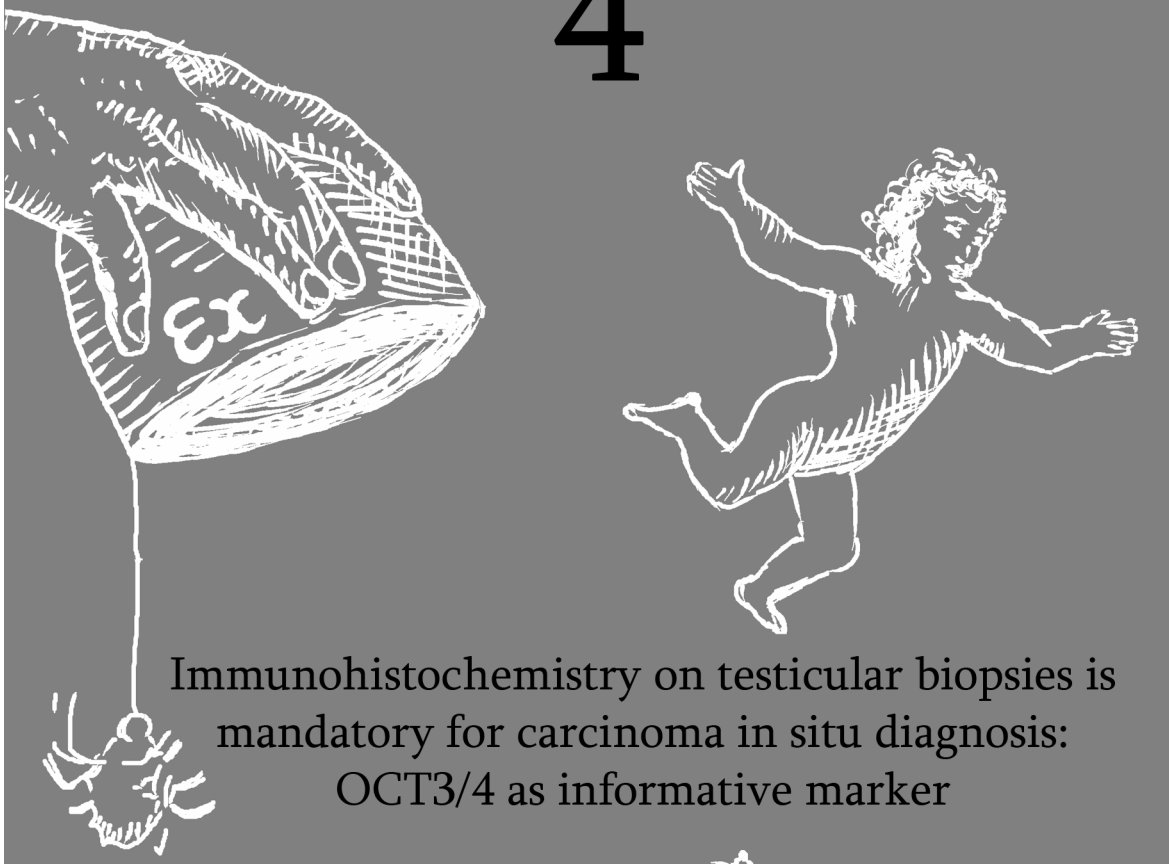

Immunohistochemistry on testicular biopsies is mandatory for carcinoma in situ diagnosis: OCT3/4 as informative marker

Immunohistochemistry on testicular biopsies is mandatory for carcinoma *in situ* diagnosis: OCT3/4 as informative marker.

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ABSTRACT

Carcinoma *in situ* (CIS) is the common precursor of all type II testicular germ cell tumours (TGCTs), i.e. seminomas and nonseminomas, the most frequent solid cancer in Caucasian adolescent and young adult males. Although CIS can be detected with a surgical testicular biopsy, the rareness of TGCTs does not justify testicular biopsies in the general population. However, specific risk factors for TGCTs have been identified, including infertility, cryptorchidism, and more recently bilateral testicular microlithiasis. Individuals with these characteristics more often have a testicular biopsy taken for diagnostic purposes than the general male population. This gives an opportunity to diagnose CIS at an early stage, allowing early treatment, thereby preventing development of an invasive cancer. However, CIS is difficult to identify using morphology alone, especially when it is focal and spermatogenesis is (still) present.

We performed a retrospective study using the Dutch pathological database (PALGA) on patients diagnosed for an invasive TGCT after orchidectomy, who earlier underwent a testicular biopsy because of infertility or microlithiasis, and signed out as non-malignant. A total of 21 men fulfilled these criteria. In 19 patients new haematoxylin-eosin (H&E) slides of the biopsies were examined using morphology alone as well as immunohistochemistry for OCT3/4 (next to PLAP and c-KIT) by a pathologist specialized in TGCTs. This revealed CIS in four cases and an invasive tumour in two (6/19=31.6%). Using immunohistochemistry, an additional four cases of CIS were identified, demonstrating an added value of immunohistochemistry of 21.1%. In three patients, this diagnosis could have prevented a second gonadectomy, leading to life long hormonal androgen substitution. Three out of nine negative biopsies contained less than 30 tubules, and should therefore have been classified as insufficient for histological diagnosis.

This study demonstrates that in a routine pathology setting immunohistochemistry is mandatory for the diagnosis of CIS of the testis. Based on staining intensity, it is recommended that OCT3/4 is applied on formalin-fixed tissue and PLAP or c-KIT on

Bouin-fixed tissue. False negative cases can be due to heterogeneous distribution of CIS in the testis, favouring taking two biopsies. Application of this protocol will allow early diagnosis, and therefore treatment by local low-dose irradiation, to prevent the development of an invasive cancer. This will save the patients the heavier treatment for an invasive cancer, and will preserve hormonal function of the treated testis in the majority of cases.

INTRODUCTION

Carcinoma *in situ* (CIS), also referred to as Intratubular Germ Cell Neoplasia Unclassified (ITGCNU) is currently accepted as the precursor of all testicular germ cell tumours of adolescents and young adults (TGCTs), i.e., the seminomas and nonseminomas, which we refer to as type II TGCTs[1,2]. The CIS cell is the malignant counterpart of a primordial germ cell/gonocyte, which develops *in utero*. The invasive TGCT will manifest itself after puberty. The life-time risk for TGCT is similar to the identified incidence of CIS in a population of young males who died of other reasons than a TGCT[3]. This indicates that all CIS will eventually progress to an invasive cancer and that no spontaneous regression occurs[1]. In other words, there is a time window for early diagnosis of CIS, which is of clinical value for various reasons. Diagnosis and treatment of CIS can prevent development of an invasive TGCTs, which carries the risk of metastasized disease, requiring irradiation or chemotherapy and sometimes extensive surgery[4]. Patients with isolated CIS can be cured using local low dose irradiation only, which spares the hormonal function of the tesis in the majority of cases[1]. Some patients at risk for type II TGCTs like those with fertility problems undergo testicular biopsies, in others a biopsy is taken to diagnose CIS, for example patients with a contralateral TGCT, bilateral testicular microlithiasis or cryptorchidism[5-11] Although CIS is virtually always found in the parenchyma adjacent to a TGCT in orchidectomy specimens, it is reported that in testicular biopsies, without an invasive tumour, CIS is missed in approximately 0.5 percent[12,13]. This has been attributed to heterogeneous distribution of CIS throughout the testis[14]. Because of this, Dieckmann *et al* proposed to take two-sited surgical biopsies from a single testis which improves the diagnostic yield with 18 percent[15]. Besides the putative heterogeneous histological distribution[16,17] CIS can also be difficult to identify in H&E stained sections when limited to a few seminiferous tubules that have retained some spermatogenesis. Recently, application of immunohistochemistry using specific antibodies has significantly increased the sensitivity and specificity of diagnosing CIS, because of the identification of (novel) highly specific and sensitive markers. OCT3/4 (POU5F1) is one of the best examples in this context[18,19]. This transcription factor is regarded as one of the key regulators of pluripotency[19,20], and inhibits apoptosis in early germ cells[21]. OCT3/4 is expressed in CIS, seminoma and embryonal carcinoma cells. All CIS, seminoma and embryonal carcinoma cells have a consistent strong nuclear staining for this marker. No OCT3/4 staining is found in normal adult testis.

In this study we assessed the additional value of OCT3/4 immunohistochemistry compared to standard morphological investigation for the diagnosis of CIS in patients with

a proven type II TGCT, who earlier underwent a testicular biopsy from the same testis because of infertility, bilateral testicular microlithiasis, or an earlier TGCT in the contralateral testis. In all cases CIS was not recognized in the biopsy. New H&E slides were examined by an expert pathologist and complemented with immunohistochemical stainings for OCT3/4, PLAP and c-KIT.

MATERIALS AND METHODS

Patient selection

Since 1971 a national pathology database (PALGA) has been used in the Netherlands, which covers all (70) academic and non-academic pathology departments. PALGA registers histo- and cytopathology using this nation-wide network.

A database search was performed to identify patients who underwent a testicular biopsy and later in life developed a TGCT. This screening resulted in 121 patients fulfilling these criteria. The findings in the original biopsies consisted of twelve cases with CIS (9.9%), 88 invasive TGCTs (72.7%; 55 seminomas and 33 nonseminomas) and 21 cases in which no malignant cells were seen (17.4%). The original paraffin blocks of the latter were collected from 10 pathology departments involved throughout the Netherlands, including a total of 28 biopsy specimens from 20 patients (nine bilateral, 11 unilateral). From one patient no material could be retrieved. The indications for performing a testicular biopsy were obtained from the original pathology report. Because of ethical restrictions, related to privacy protection, to the use of data derived from PALGA, it was not possible to obtain additional patient information, other than mentioned in the pathology report.

Immunohistochemistry & evaluation

Three micron thick tissue sections were cut of all the retrieved blocks and stained with haematoxylin and eosin (H&E). The slides were evaluated by a pathologist experienced in TGCT pathology (J.W.O.). In addition, parallel sections were stained using immuno-histochemistry for c-KIT, PLAP, and OCT3/4, as described before [18] . Endogenous peroxidase activity was inactivated by incubation in 3% H_2O_2 for 5 min. Antigen retrieval was carried out by heating sections in 0.01 M sodium citrate (pH 6.0) for formalin-fixed tissue or 0,001M EGTA/0,01M Tris/HCl (pH9.0) for Bouin-fixation, under high pressure till 1.2 bar for all three antibodies. Endogenous biotin was blocked using an avidin/biotin blocking kit (SP-2001; Vector Laboratories, Burlingame, CA, USA). For OCT3/4 immunohistochemistry sections were incubated for 2 h at room temperature with a monoclonal OCT3/4 antibody (sc-5279; Santa Cruz Biotechnology, Santa Cruz, CA, USA) directed against the NH_2 terminus of the protein. The OCT3/4 antibody was diluted 1:1000. Subsequently, a biotinylated rabbit anti-mouse secondary antibody (E0413; Dako, Glostrup, Denmark) diluted 1:200 was applied and bound antibody complex was visualized using the horseradish peroxidase (HRP) avidin-biotin complex method. Sections were counterstained with haematoxylin. PLAP and c-KIT immuno-histochemistry was performed as described earlier[22].

The biopsy samples were blinded prior to evaluation, and the presence of CIS was scored first on the H&E stained sections and subsequently on the OCT3/4, PLAP and c-KIT stained slides. These immunohistochemically stained slides were compared to asses differences in staining pattern between these techniques.
To asses if a reliable diagnosis could be made, according to the recommendations of Nieschlag[23], the total number of seminiferous tubules was counted. The orchidectomy specimens were diagnosed according to the 2004 WHO classification[24].

RESULTS

In 21 out of the 121 patients (17.4%) morphological investigation of an H&E stained histological section of the testicular biopsy performed in a routine pathology department revealed no CIS or invasive type II TGCT, although all 21 subsequently developed an ipsilateral TGCT, confirmed by orchidectomy specimen evaluation. The biopsies were taken between 1981 and 2003, and the mean age at time of biopsy was 29.1 years (range 16 to 36 years). The biopsy indication is shown in Table 1. The mean age at time of orchidectomy was 33.2 (range 19 to 44 years), and the mean time span between the original biopsy and the orchidectomy in these patients was 52.4 months (range 9.3 to 156.5 months). We were able to obtain paraffin blocks from twenty patients. In one of these the paraffin block lacked residual material. In total we were able to re-evaluate testicular biopsies from 19 patients. Pathological examination of the 21 orchidectomy specimens revealed 13 seminomas, six nonseminomas, one CIS-only and one burned out tumour with CIS in the adjacent parenchyma (Table 1). Four patients had a history of unilateral TGCT, in three of them a biopsy was performed because of infertility.
Re-evaluation by an expert in germ cell tumour pathology (J.W.O.) showed, based on morphological examination of newly cut slides of the same paraffin blocks used for the initial evaluation, that malignant cells were present in six out of the 19 patients (31.6%, i.e., four CIS (one of them with a previous TGCT), and two invasive seminomas. In three other biopsies (15.8%) the histology was recognized as suspicious for the presence of CIS, although it could not be diagnosed with certainty. Immunohistochemistry was deemed necessary before coming to a definitive diagnosis. In addition to the six cases diagnosed on H&E morphology alone, immunohistochemistry for OCT3/4, PLAP and c-KIT identified four more cases (10 out of 19 cases, 52.6%), including the three cases identified as suspected. In other words, an experienced pathologist in TGCT pathology is able to correctly diagnose CIS in 60.0% of the cases and 90.0% including those classified as suspicious. One patient (case no. 7 in Table 1; Figure 1) underwent three successive biopsies within a period of 1.5 year for unknown reasons. While based on the H&E stained sections no CIS was identified, both originally and in the review, OCT3/4 immunohistochemistry demonstrated CIS in the second and third biopsy. In four cases, the biopsy samples (patients no. 1, 8, 11, and 15 in Table 1) were negative for CIS on H&E evaluation and contained 30 tubules or less. Therefore, these biopsies are in principle not appropriate to allow diagnosis, because of the risk of false negative findings. However, immunohistochemistry demonstrated CIS in one of them.

TABLE 1. Patient characteristics with initial pathological evaluation and revision results.

Case no.	Age at biopsy (years)	History	Fixative	Age at orchidectomy	Initial biopsy diagnosis	New H&E	Discordance initial diagnosis and new H&E	OCT3/4	Discordance new H&E and OCT3/4	Orchidectomy diagnosis
1	36	azoospermia	F	38	bilateral JS 8-9	no CIS		-		L: SE
2	32	bilateral orchidopexy azoospermia	B	39	R: hypospermatogenesis, some SCOS tubules, Leydig cell hyperplasia	no CIS		-		R: EC + TE
3	31	orchidopexy, infertility, oligospermia	B	32	R: SCOS, focal hypospermatogenesis JS 2-3	suspect		+		R: SE
4	35	azoospermia	B	44	bilateral hypospermatogenesis	no CIS		-		R: SE
5	28	R: orchidopexy , azoospermia	B	39	bilateral JS 9-10	no CIS		-		R: SE
6	27	infertility	F	39	bilateral JS 3	CIS L + R	+	L : +, R : +		R: SE
7	30	R: orchidectomy chemotherapy BEP + RPLND, azoospermia	B+F	33	3 biopsies: maturation arrest	1: no CIS 2: no CIS 3: no CIS		1: -, 2: +, 3: +	+	L: SE
8	33	-	F	34	bilateral JS 9-10	no CIS		L: -		L: SE
9	28	azoospermia	F	34	bilateral SCOS, severe spermatogenesis arrest	R: micro invasive SE	+	R: +		R: SE
10	34	orchidopexy, azoospermia	B	37	bilateral oligospermia, atrophy, Leydig cell hypoplasia	no CIS		-		R: TE
11	29	bilateral testicular microlithiasis	B	29	bilateral JS 10	no CIS		-		L: SE
12	28	R: orchidectomy for TGCT, infertility	F	31	L: hypospermatogenesis, Leydig cell hyperplasia	CIS	+	+		L: SE
13	24	R: NS, azoospermia	F	28	L: 30 % SCOS, some spermatozoa	suspect		+		L: SE
14	34	azoospermia	F	38	L: JS 9	no CIS		-		L: SE
15	28	-	F	29	R: fibrosis, necrosis	no CIS		-		R: EC + YST
16	31	infertility	F	34	bilateral JS 7	CIS	+	L: -, R +		R: SE
17	31	azoospermia, normal gonadotrophins	F	36	L: maturation arrest	CIS	+	+		L: CIS
18	29	-	.	30	R: infection, suspicious for sarcoidosis	no material				R: NS
19	29	-	B	33	R: JS 2, areas with JS 6-7	no material				R: CIS + burned out SE
20	16	R: TE	F	19	L: JS 10	suspect		+		L : NS
21	21	-	F	21	L: reactive connective tissue, no testis in this biopsy	NS	+	+		L : NS

Legend: JS=Johnsen score; SCOS=Sertoli cell only syndrome; BEP=Bleomycin/Etoposide/Cisplatin; RPLND=Retroperitoneal Lymph Node Dissection; F=formalin ; B=Bouin's fixative ; L=left ; R=right; CIS=carcinoma in situ; NS=nonseminoma; EC=embryonal carcinoma; SE=seminoma; YST=yolk sac tumour; TE=teratoma

In our study Bouin-fixated biopsy specimens showed a weaker nuclear OCT3/4 staining compared to formalin-fixated specimens (data not shown). This is in contrast to our earlier study, in which no difference of fixative on OCT3/4 immunohistochemistry was found[18]. Most likely this is due to the duration of fixation. This was not seen for both the non-

nuclear markers c-KIT and PLAP. In spite of the weaker staining intensity for OCT3/4, no difference was seen in the amount of CIS identified compared to c-KIT and PLAP.

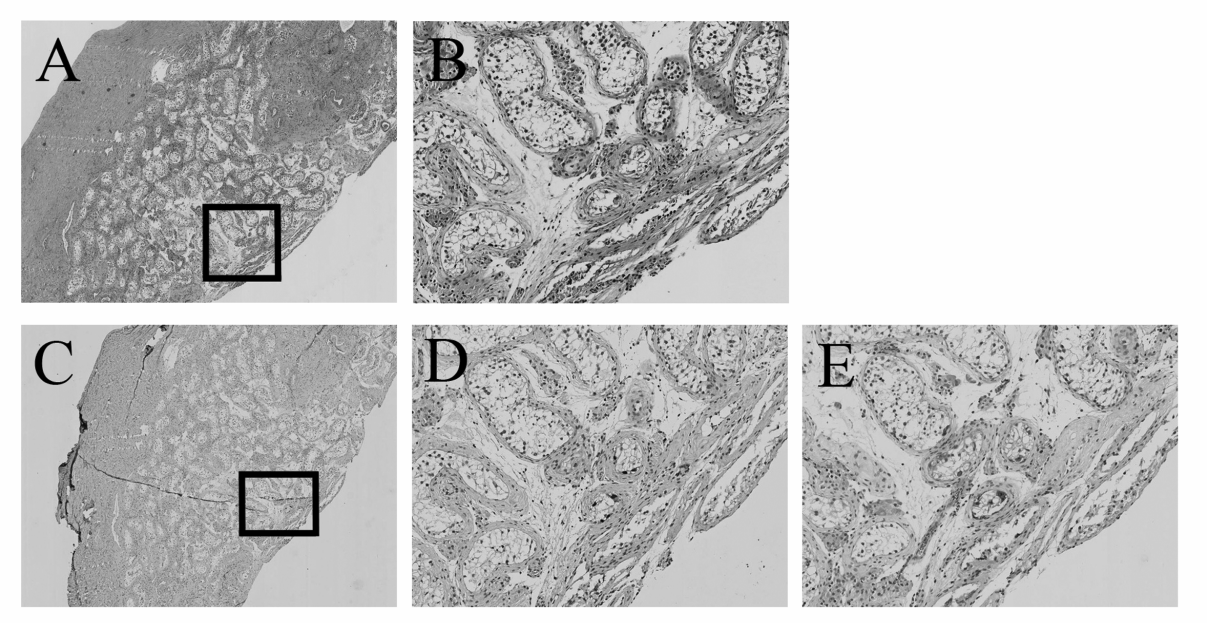

FIGURE 1. A) Histology of an open surgical biopsy of the testis stained with H&E (25x) (patient 7, Table 1). It was identified as suspected, but not proven to contain CIS; B) Higher power of suspected lesion (H & E 100x); C) Histology of the same biopsy stained immunohistochemical for OCT3/4 (25x). The region with positive cell is indicted in the square; D) Higher power of suspected lesion (OCT3/4, 100x); Only three tubules contained OCT3/4 positive cells (brown); E) PLAP immunohistochemistry of the same region, confirming the presence of CIS cells (red). *(Figure 1 colour image on page 181)*

Twelve out of the 121 patients (9.9%) were correctly diagnosed with CIS on the testicular biopsy during the initial morphological investigation using morphological analysis alone, and were subsequently treated by orchidectomy. These patients had a mean age of 28.1 year at time of orchidectomy (range 18-38). The mean age of patients at time of orchidectomy diagnosed with CIS and of those in whom CIS was initially missed was not significantly different, respectively 28.7 vs. 28.1 (p=0.77).

DISCUSSION

This is the first study to describe the additional value of immunohistochemistry for diagnosing CIS in testicular biopsies in a nation-wide setting. The findings are in line with the conclusions based on epidemiological observations that CIS will always develop into an invasive tumour, and no spontaneous regression occurs[25]. Therefore, diagnosis of CIS is an indication for treatment. Our study shows that evaluation of standard H&E stained section of biopsy material, CIS is overlooked and that the use of immunohistochemistry is mandatory.

In 1983, placental like alkaline phosphatase (PLAP) was the first marker used in the detection of seminomas. Since then other markers as c-KIT, AP_2-gamma and OCT3/4

followed[20,26-28]. Currently, OCT3/4 is best characterized and a reliable marker in TGCT diagnostics and 100% of the cells of CIS, seminoma and embryonal carcinoma have strong nuclear staining[18,19,29]. In contrast to PLAP which is positive in 93-98% of the cases and the frequent finding of only focal c-KIT positivity in CIS[29,30], Jones *et al* reported on the extra diagnostic yield of OCT3/4 staining in a retrospective study in 6 out of 157 patients[31]. Two out of these six patients were younger than 16 months and therefore the clinical importance of this finding can be questioned, as OCT3/4 positive cells found in normal testis of neonates[32,33] do not always implicate neoplastic transformation of germ cells. Our study, including clinical follow-up, is based on revision of testicular biopsies of 19 patients who developed a TGCT after an initial CIS negative biopsy. By examination of H&E stained slides alone six biopsies were scored positive for CIS or invasive TGCT (31.6%). Additional immunohistochemistry for OCT3/4, PLAP and c-KIT identified four additional positive biopsies making an extra diagnostic yield of 21.1 percent. Our results show that 10 out of 19 biopsies (52.6%) were initially incorrectly diagnosed and that additional immunohistochemistry is mandatory to come to a correct diagnosis. The development of a clinically manifest tumour is the only method to absolutely proof that a biopsy is false-negative. Therefore follow-up data is crucial in accurately defining the extra diagnostic yield of immunohistochemistry of biopsy specimens.

Immunohistochemical staining results are dependent on tissue fixation and standardization of the protocols. Bouin's fixative is known to provide excellent nuclear morphology, which is achieved by the addition of picric acid to formalin, but this fixation results in prevention of DNA denaturation[34]. We speculate that this could affect the immunoreactivity of the OCT3/4 nuclear antigen and cause sub-optimal immunohistochemical staining. Guidelines concerning fixatives of testicular biopsies are contradicting on the use of Bouin's fixative or formalin[4,35]. Winstanley *et al* [35] suggested to use formalin fixation in case of CIS diagnostics and Bouin's fixative in assessing spermatogenesis. In addition, the use of Bouin's fixative makes DNA and RNA retrieval for molecular analysis troublesome, which may hamper additional molecular studies.

CIS was thought to be homogeneously distributed throughout the testis and therefore be diagnosed with only a single open surgical biopsy containing >30 tubules and at least 3 mm in diameter[1,23]. However, various orchidectomy studies revealed that CIS is non-randomly distributed throughout the testis and false negative biopsy results can occur[16, 36]. Dieckmann *et al* recently advised to perform a two-sided testicular biopsy, which in his hands increases the diagnostic yield with 18%[37]. In our cohort six testicular biopsies from four patients contained less than 30 tubules and, therefore, a reliable negative diagnosis cannot be made. In spite of the limited amount of material available, OCT3/4 immunohistochemistry showed the presence of CIS in one biopsy. In conclusion, as recommended as standard, physicians should take responsibility to provide an adequate biopsy containing at least 30 seminiferous tubules, allowing a reliable diagnosis. In this study, no correlation between presence or absence of CIS in the biopsy and age of the patient was found.

Recent studies have shown a prevalence of CIS in infertile men of approximately 1% to 5%[1,38]. In this perspective it is striking that testicular biopsies of men undergoing a testicular biopsy for evaluation of spermatogenesis or sperm extraction (TESE) are not

routinely checked for CIS. Schulze *et al* found five patients with CIS (two bilateral) out of 766 patients undergoing TESE (0.7%)[39]. Only two articles describe histological evaluation of TESE material and the presence of malignancy, based on morphological criteria alone[39,40]. Both studies underscore the need for histological evaluation for patients at risk for TGCT who have a TESE performed. Other risk factors include a history of undescended testis, ambiguous genitalia, contralateral TGCT or bilateral testicular microlithiasis, with an estimated prevalence of 3, 6-25, 5 and 20%, respectively[1,11]. In these men testicular biopsies should be routinely checked on CIS besides scoring quality and quantity of spermatogenesis. It is the responsibility of the referring physician to accurately judge the risk for CIS and inform the pathologist of the risk factors in a particular patient.

Diagnosis of CIS before development of an invasive tumour protects patients from disseminated disease. Metastatic disease often needs treatment regimes containing radiation or chemotherapy, which may lead to severe long-term sequelae. Moreover, there is no quarantee that CIS present in the contralateral testis will be eradicated by chemotherapy. Therefore, a secondary germ cell tumour can still develop[41]. In contrast to invasive tumours, CIS is curable in almost 100%, although Dieckmann *et al* reported on two cases of testicular cancer despite previous radiotherapy (20 Gy) to the testis[42]. Testicular irradiation eradicates all germ- and CIS cells, but may effect Leydig cell function and induce hypogonadism in only a limited number of cases[43]. However, this stands in no contrast to the potential side-effects of chemotherapy, such as vascular toxicity, peripheral neuropathy and the potential formation of secondary tumours[41,44]. In our study four patients with a history of TGCT developed a contralateral tumour after a false-negative biopsy. In all cases review of the initial biopsy showed the presence of CIS. Treatment of CIS in these three patients with local radiation would have prevented the second orchidectomy and life-long treatment with androgens. At least one of these three patients was initially treated with chemotherapy, confirming that receiving chemotherapy (bleomycin/etoposide/cisplatin) does not rule out development of a contralateral cancer. Christensen *et al* found CIS in the contralateral testis after chemotherapy in six out 33 patients and estimated a cumulative risk at 10 years of 42%[45]. It is suggested that patients with contralateral CIS should be treated with localized radiotherapy or Carboplatin based chemotherapy[45,46].

We are aware of the limitations of our retrospective study. No data are available on treatment modality, survival and quality of life. Therefore, we cannot make a definite calculation regarding the profit of making a diagnosis of CIS in the first biopsy. However, we can conclude that these patients at least were spared an orchidectomy if they would have been treated with irradiation alone and life-long androgen supplementation would have been prevented in a selection of them.

A number of conclusions can be drawn from this study: 1) immunohistochemistry is needed for the diagnosis of CIS on testicular biopsies, even for expert pathologists; 2) OCT3/4 is recommended on formalin-fixed tissue, and c-KIT or PLAP on Bouin-fixed tissue (depending on fixation duration); 3) physicians must be aware of the risk factors for type II TGCTs, and provide these as relevant clinical information in the pathology request

form; 4) testicular biopsies containing at least 30 seminiferous tubules are needed for accurate diagnosis of CIS.
Our study shows that immunohistochemistry is mandatory in evaluating testicular biopsies for the presence of CIS.

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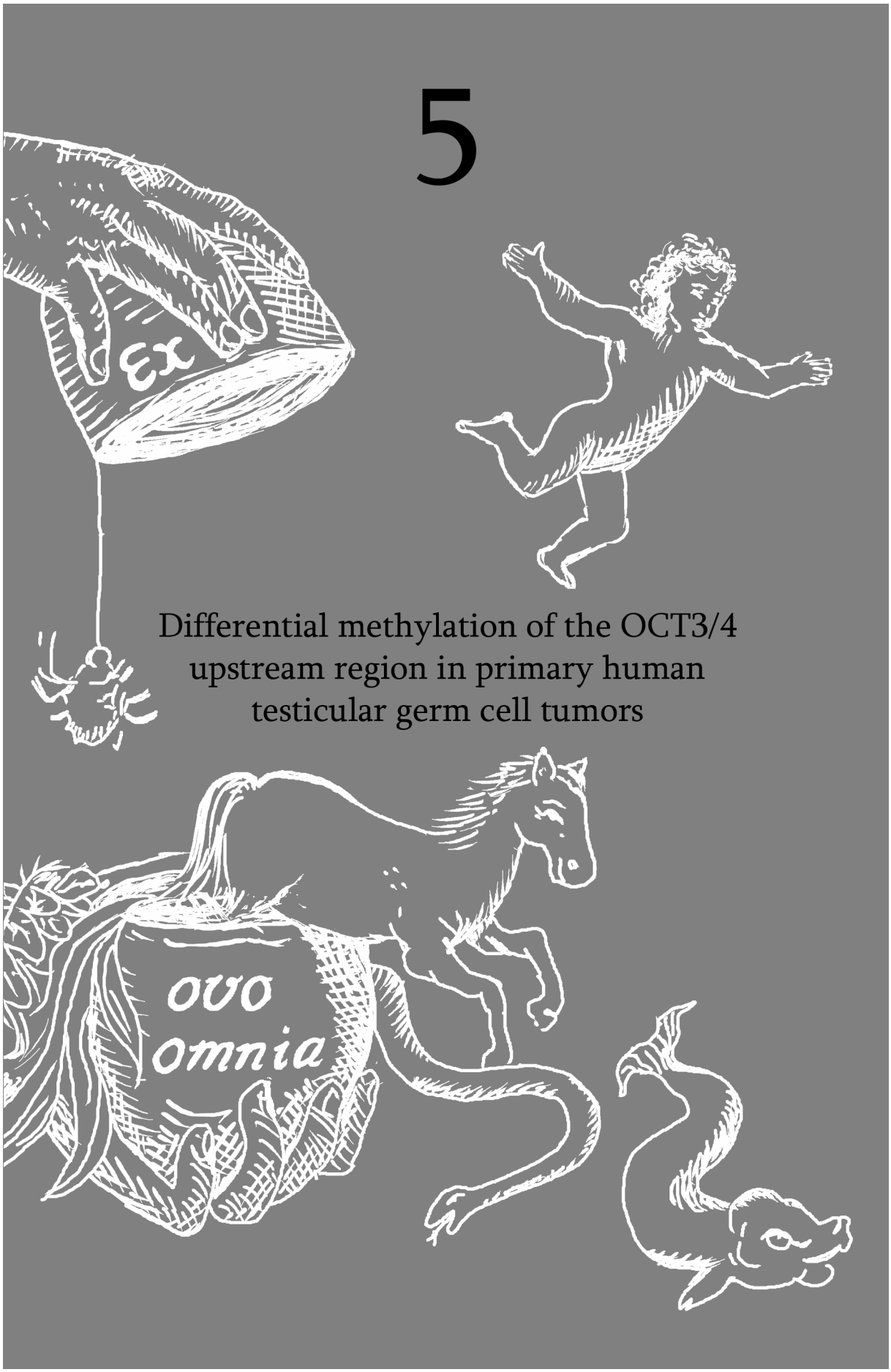

Differential methylation of the OCT3/4 upstream region in primary human testicular germ cell tumors

Differential methylation of the *OCT3/4* upstream region in primary human testicular germ cell tumors

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ABSTRACT

Germ cell tumors show many similarities to normal embryogenesis. This is, for example, illustrated by the expression of the marker of pluripotency, *OCT3/4*, known to play a pivotal role in the early stages of normal development. Interestingly, it is found to be the most informative diagnostic marker for the early developmental stages of malignant germ cell tumors. Expression regulation of *OCT3/4* has been extensively studied in murine and human cell lines, including embryonic stem cell lines and tumor derived cell lines. We investigated for the first time the methylation status of the upstream region of the *OCT3/4* gene in normal and neoplastic testicular primary tissues using bisulfite genomic sequencing. The cell line JKT-1, supposedly seminoma-derived, was included in the survey. Normal testis parenchyma, peripheral blood lymphocytes, spermatocytic seminoma, yolk sac tumor and teratoma, and JKT-1 showed a consistent hypermethylation. In contrast, seminoma and embryonal carcinoma were hypomethylated, confirmed by analyses after tumor micro-dissection. Testicular lymphomas showed the most heterogeneous pattern, although specific regions were consistently hypermethylated.

In conclusion, the results obtained from this set of adult normal and neoplastic *in vivo* derived samples is in accordance to the *in vitro* data that expression of *OCT3/4* is associated with specific changes in methylation. Moreover, the findings argue against OCT3/4 being a driving oncogenic factor in the pathogenesis of human germ cell tumors.

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INTRODUCTION

Gene expression during normal development is tightly regulated by epigenetic modifications, including cytosine (CpG) methylation and histone acetylation (1). During the process of tumorigenesis, the physiological (normal) epigenetic patterns may become disturbed and as a result, genes under control of these epigenetic modifications, may become silenced by promoter hypermethylation (2). Testicular germ cell tumors of adolescents and young adults (TGCTs), i.e., the seminomas and non-seminomas, have a heterogeneous epigenetic phenotype (3). Overall, gene promoters in seminomas are highly demethylated, whereas the non-seminomas show methylation of the majority of promoters, as reported for other solid tumors (4,5). The methylation status of seminomas is consistent with the cell of origin of TGCTs, the primordial germ cell (PGC) (reviewed in ref. 3). During early embryogenesis, PGCs migrate from the yolk sac to the genital ridge, from which the gonad develops. During this migration the epigenetic marks of the PGCs are largely erased and only become re-established as the PGCs mature during gametogenesis eventually leading to either oocytes or spermatozoa (6). Another hallmark that PGCs share with some histological elements of TGCTs is the expression of *OCT3/4* (*POU5F1*), a member of the POU family of transcription factors. *OCT3/4* is regarded as one of the key regulators of pluripotency and is only expressed in pluripotent stem and germ cells (7-10). In the human embryo, *OCT3/4* expression starts at the morula stage and becomes restricted at first to the inner cell mass during blastocyst formation, subsequently to the epiblast during gastrulation and finally to the PGCs. In TGCTs mRNA and protein encoded by *OCT3/4* is present in carcinoma *in situ* (CIS), the precursor of all TGCTs (11), as well as in all seminomas and the undifferentiated component of non-seminoma, i.e., embryonal carcinoma (EC) (12,13). Yet, when EC cells differentiate, either into teratoma, yolk sac tumor or choriocarcinoma, expression of *OCT3/4* is consistently turned off (12). In murine and human EC-derived cell lines the same pattern of down-regulation is found upon induction of differentiation by retinoic acid (14,15). However, it must be emphasized that murine and human EC cells are not interspecies equivalents and have a different pathogenesis (3).

Methylation of the *OCT3/4* upstream region has been studied in several human and murine tumor-derived and embryonic stem cell (ESC) derived cell lines. In mouse ESCs, Hattori *et al* showed the hypomethylation of this region and compared it to trophoblast stem cells (TSCs) and mouse liver cells which were hypermethylated (16). In addition, they showed that *in vitro* methylation suppressed *OCT3/4* enhancer/promoter activity in a reporter assay, and the demethylating agent 5-aza-2'-deoxycytidine induced aberrant *OCT3/4* expression in TSCs. In cloned mouse embryos the *OCT3/4* promoter underwent gradual demethylation during pre-implantation development and inefficient demethylation of the *OCT3/4* promoter was associated with developmental retardation at early cleavage stages (17). In the malignant equivalent of ESCs, the EC cells, it was demonstrated in 1993 that inhibition of *OCT3/4* expression in retinoic acid (RA)-induced differentiated murine EC cells is achieved through changes in methylation status, chromatin structure and transcriptional activity of the *OCT3/4* upstream regulatory region (18). These results were confirmed by independent studies (19,20). Loss of *OCT3/4*

expression during differentiation of a human EC-derived cell line (NTera2) was found to be correlated with increased methylation in the *OCT3/4* upstream region (15). Recently, this correlation was also found in human ESCs (21).
To augment the current knowledge, we analyzed the methylation status of the 2.3-kb upstream region of the human *OCT3/4* gene in different types of primary TGCTs and nongerm cell tumors of the testis. Analysis was performed by PCR and sequencing of bisulfite-treated DNA from seminoma, histological variants of non-seminoma, spermatocytic seminoma, testicular lymphoma, normal testicular parenchyma and peripheral blood lymphocytes. In addition, micro-dissected seminoma and EC as well as the cell line JKT-1, supposedly derived from a seminoma (22), were included. We demonstrate a tissue-specific differential methylation of the upstream region of the *OCT3/4* gene correlating with gene expression, with a profound general hypomethylation in microdissected seminoma and EC and a heterogeneous hypermethylation in samples of differentiated histology. Some regions were identified that could be related to activity of the promoter.

MATERIALS AND METHODS

Tissue samples

Use of tissues for scientific reasons has been approved by an institutional review board (MEC 02.981). The samples are used according to the 'Code for Proper Secundary Use of Human Tissue in the Netherlands', as developed by the Dutch Federation of Medical Scientific Societies (FMWV) (version 2002).

Immunohistochemistry

Frozen sections were stained using immunohistochemistry with a monoclonal antibody for OCT3/4 (sc-5279; Santa Cruz Biotechnology) as described previously (23). The secondary antibody was a biotinylated rabbit anti-mouse antibody (E0413; Dako). Final visualization was made with diaminobenzidine (DAB, FLUKA), after which the slides were counterstained with haematoxylin.

DNA-isolation and PCR

Genomic DNA was isolated from peripheral blood lymphocytes, normal testis parenchyma, spermatocytic seminoma, testicular lymphoma, embryonal carcinoma, yolk sac tumor, teratoma and seminoma by standard phenol extraction. Of each type of material three independent DNA samples were used, except for yolk sac tumor of which only two samples were included. In addition, genomic DNA from the JKT-1 cell line was isolated. DNA was treated with bisulfite (EZ DNA methylation kit, Zymo Research) to convert unmethylated cytosines into uracil. On this converted DNA PCR was performed with seven primer pairs used in previous experiments (15). For the PCR reactions two different Taq polymerases were used. For primer pair 3 and 4 Taq polymerase (Qiagen)

was used with the following program on a MJ Research Cycler (Waltham): 95°C for 3 min, 35 cycles of 94°C for 30 sec (denaturation), 58°C for 45 sec (annealing), 72°C for 1 min (extension), and finally an extension at 72°C for 8 min. For primer sets 5-9 a High Fidelity Taq polymerase (Invitrogen) was used with this program: 95°C for 3 min, 35 cycles of 94°C for 30 sec, 58°C for 45 sec, 68°C for 45 sec, and an extension at 68°C for 8 min. The obtained PCR products were purified (Qiagen PCR-28106 purification kit).

Sequencing

Forward primers were labelled with [γ32P]ATP. PCR products were sequenced using a cycle reader DNA sequencing kit (K1711 Fermentas). The sequencing program was 95°C for 3 min, 25 cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 1 min and 4°C for 30 min. Gels (6 and 8%) ran from 1-4 h, depending on the size of the sequenced fragment.

Micro-dissected samples

Micro-dissection was performed with a PALM-microlaser system (Zeiss) on frozen slides guided by Alkaline Phosphatase staining found to be informative to distinguish seminoma and EC cells from other cell types (24,25). DNA from three micro-dissected ECs and seminomas was processed as described above by bisulfite treatment, PCR and sequencing.

RESULTS

We examined the methylation status of 32 CpG sites between the transcription start site and position -2351 upstream of the human *OCT3/4* gene of *in vivo* derived human samples. In addition, matched micro-dissected samples were included to exclude misinterpretation of the data due to the presence of host tissue (Fig. 1). Based on sequence analysis each position was scored as complete methylation, partially methylated and unmethylated (Fig. 2). Because direct sequencing was applied to the tumor samples, we checked if this method was comparable to sequencing of cloned PCR products. Therefore, part of the upstream sequence (primer pair 9) was analyzed by both methods in undifferentiated and retinoic acid differentiated NTera2 cells. Results were similar [data not shown, in accordance to previous findings (26)] and confirmed the results obtained by Deb-Rinker *et al* (15). Therefore, we only investigated the direct sequencing derived data in this study. Results from the human tissue samples are schematically represented in Fig. 3.

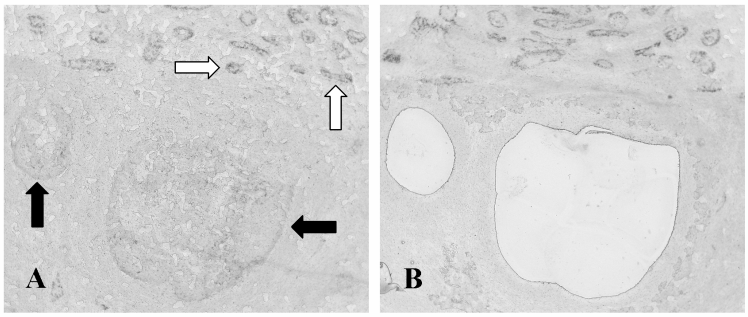

FIGURE 1. Embryonal carcinoma (black arrows) before (A) and after (B) micro-dissection. Enzymatic alkaline phosphatase stains embryonal carcinoma as well as the carcinoma *in situ* cells (white arrows).
(Figure 1 colour image on page 181)

Some of the positions could not be reliably scored due to sequencing limitations and these are omitted from further analysis as indicated (Fig. 3). The CpG sites at position -1891 and -2351 could not be conclusively analyzed in any sample. Deviating from the sequence of the upstream region of the human *OCT3/4* gene in the article by Nordhoff *et al* (27) we found two extra CpG sites at positions -1692 and -1755 generated by single nucleotide polymorphisms. For position -1692 the polymorphism was CG/CA and for position -1755 it was CG/CT. In addition, position -1772 was also a polymorph and consisted of a CG/CC polymorphism. Frequency and functionality of these specific polymorphisms in normal or patient populations were not investigated in this study, and remain to be elucidated.
To compare the methylation status of the different samples, the number of complete methylated CpG sites was divided by the total number of CpG sites analyzed. This resulted in the percentage of completely methylated CpG sites per sample (Fig. 3). Peripheral blood lymphocytes, spermatocytic seminoma and differentiated components of non-seminoma, i.e., yolk sac tumor and teratoma had a hypermethylated upstream region of the *OCT3/4* gene, ranging from 56 to 89%. The testicular lymphomas were heterogeneous with methylation percentages of 31 to 94%, while they were all immunohistochemically negative for OCT3/4. The JKT-1 cell line was hypermethylated, and also lacked expression of OCT3/4.
In contrast to the differentiated TGCT components, the seminomas and embryonal carcinomas had a relatively low level of CpG methylation, ranging from 9 to 42%. Matched micro-dissected samples of these two histologies, lacking (hypermethylated) host cells, such as lymphocytes and normal testis parenchyma, were all unmethylated (Fig. 3).

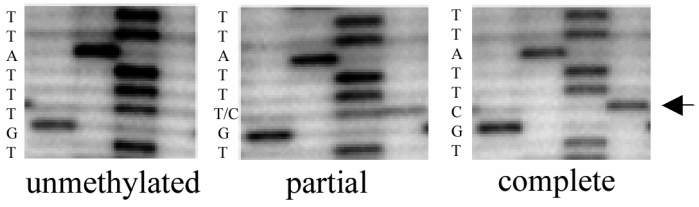

FIGURE 2. Sequencing analysis of bisulfite treated DNA. Position-1755 (arrow) in representative samples. Unmethylated CpG site shows only a band at the T position (left panel), partially methylated site has a band at both the T and C position (middle panel), and complete methylated site has only a band at the C position (right panel).

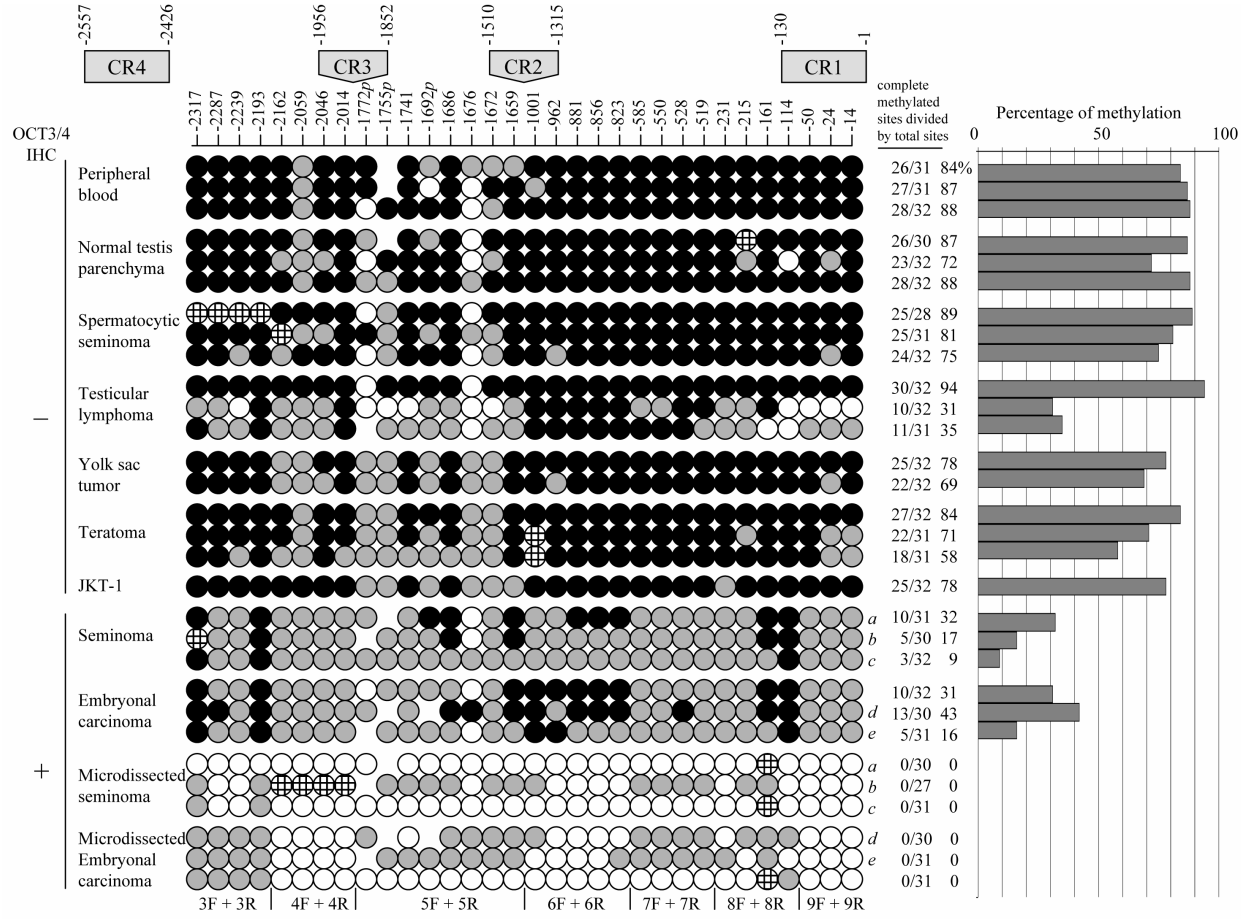

FIGURE 3. Methylation status of the upstream region of the *OCT3/4* gene. At the top conserved regions (CR) between human, murine and bovine sequences are represented as grey boxes and accompanied by their relative position to the transcription start site (TSS). Directly below the examined CpG sites and their position relative to TSS are indicated. On the left side are the histological groups, each group consisting of three independent DNA samples, except for the group of yolk sac tumors that contains two samples. In addition, OCT3/4 immuno-histochemistry (IHC) results are indicated as positive (+) or negative (-). Each horizontal row of circles represents one sample. A black circle signifies a complete methylated CpG site, a grey circle partial methylation and a white circle no methylation. In addition, gritted circles signify an inability to analyse and polymorphic sites (indicated by *p*) not constituting a CpG are omitted. Letters *a* to *e* at the right end of (micro-dissected) seminoma and embryonal carcinoma samples indicate which samples were derived from the same tumor. The primer pairs that cover the CpG sites are represented at the bottom. At the right of each row is the fraction of complete methylated CpG sites and total CpG sites analysed. This fraction is represented as a percentage in the vertical histogram. Differentiated samples of peripheral blood lymphocytes, normal testis parenchyma, spermatocytic seminoma, yolk sac tumor and teratoma have a high level of methylation. Seminoma and embryonal carcinoma were hypomethylated, which was confirmed in the micro-dissected samples where no contaminating normal cells were present. In contrast, methylation in the group of testicular lymphomas was heterogeneous.

DISCUSSION

The methylation status of the upstream region of the *OCT3/4* gene has been studied in various murine and human cell lines, both benign and malignant. Here we showed the differential methylation of this region in a range of primary human testicular tumors and normal tissue, an unexplored field so far. This differential methylation is in accordance with previous data obtained from *in vitro* grown cells. In fact, undifferentiated, *OCT3/4* expressing cells, have overall a hypomethylated upstream region, whereas differentiated cells, lacking OCT3/4 expression, are hypermethylated.

In our study, seminoma and the undifferentiated nonseminomatous EC, both characterized by OCT3/4 expression, are hypomethylated. However, tumor tissue can be quite heterogeneous and especially seminoma cells are intermingled with lymphocytic infiltrations that can drastically decrease the percentage of tumor cells to below 50%. Also, EC often constitutes a low percentage of cells in the tissue, because of growing in nests and their capacity to differentiate into extra-embryonic and embryonic tissues. Of course, this contamination distorts the results of the analyses, especially if total samples are investigated. Therefore, in this study, also micro-dissected seminoma and EC samples were investigated, and these samples showed almost no methylation of the region analyzed. In contrast to seminoma and EC, differentiated non-seminomatous components, teratoma and yolk sac tumor, which do not express OCT3/4, were indeed found to be hypermethylated. Spermatocytic seminoma, normal testis parenchyma and peripheral blood lymphocytes, all consisting of differentiated cells, are hypermethylated. These findings support the different cell of origin and pathogenesis of seminoma and spermatocytic seminoma (28). The cell line JKT-1, supposed to be derived from a seminoma (22), was hypermethylated (78%), adding to the accumulating evidence that it is not of seminomatous origin (unpublished data). The testicular lymphomas are the only tumors that show a heterogeneous pattern in this limited set of samples. While all were negative for OCT3/4, two (both of T-cell origin) showed a low level of methylation, while one B-cell lymphoma showed a high level of methylation. This might be of interest, although it needs further investigation (29).

In the human EC-derived cell line NTera2 Deb-Rinker *et al* showed that a relatively small increase in methylation of specific CpG sites in the upstream *OCT3/4* region is sufficient to shut-down expression of the gene (15). These specific CpG sites correspond to positions -1001, -231, -50, +5 and +35 (Fig. 3). The sites upstream of the transcription start site were also differentially methylated in our analysis; the latter two were not represented in our search. In addition, the previous report identified CpG sites that were already methylated in the undifferentiated EC cells. This block of constitutive methylation (CpG sites -519 to -881) was not present in the microdissected EC samples and is possibly related to the clonal origin of the NTera2 cells and/or their *in vitro* propagation. It is indeed known that upon *in vitro* growth hypermethylation might occur compared to the original *in vivo* obtained cells (30).

Expression of the *OCT3/4* gene is, besides methylation, regulated by other factors, such as binding of transcription factors to specific regions in the upstream sequence. These regions are conserved between different species and designated CR1 to CR4 by Nordhoff

et al (27). OCT3/4 expression is regulated by a minimal TATA-less promoter (31) present in CR1. In addition, there are two enhancer sites more upstream: a proximal enhancer (PE) mainly coinciding with CR2 that activates *OCT3/4* in the epiblast (32), and a distal enhancer (DE) in CR4 driving gene expression in the morula, inner cell mass and PGCs (32). Next to the DE the CR4 also contains the OCT3/4-SOX2 binding site (33). These promoter and enhancers are the key regulatory sequences for *OCT3/4* gene expression and therefore methylation status of their associated CpG sites can be expected to be of more importance than the sites of non-regulatory upstream sequences. However, thus far this was not experimentally tested. The minimal *OCT3/4* promoter in CR1 has no CpG island, but harbors 4 of the investigated CpG sites (position -14, -24, -50 and -114 relative to the transcription start site). Unfortunately, the DE in CR4 lies outside the examined upstream region and CR2 containing the PE has no CpG sites (Fig. 3). Because of the limited number of CpG sites in the regulatory regions, no clear relation between methylation of these regions and gene expression could be made. However, some general patterns can be observed. Overall, CpG sites -14 to -1659 and -2193 to -2317 show differential methylation between differentiated and undifferentiated tissue, whereas this relation is not present in CpG -1672 to -2162 lying in between (Fig. 3). The same pattern was observed in NTera2 cells (15). Most of these differentially methylated CpG sites had no association with known transcription factor binding sequences, except for position -114 where Sp1 and Sp3 can bind.

Genomic methylation patterns in solid tumors might be disturbed compared to their normal counterpart, causing for example gene silencing by aberrant promoter methylation (34). If this is the case, these epigenetic alterations, together with mutations, can contribute to the process of tumorigenesis (reviewed in ref. 34). Thus far, no mutations or aberrant epigenetic patterns for *OCT3/4* have been described and *OCT3/4* is only expressed in the malignant counterpart of cells normally expressing the gene, i.e., CIS/seminoma and EC, respectively representing neoplastic PGCs and ESCs (35). This observation, together with our results presented here on the methylation status in human TGCTs supports the view that *OCT3/4* is not one of the driving oncogenic forces in these tumors. However, the data support the model that TGCTs can be used to study mechanisms involved in normal embryogenesis, as we previously reported for X-inactivation (36).

ACKNOWLEDGEMENTS

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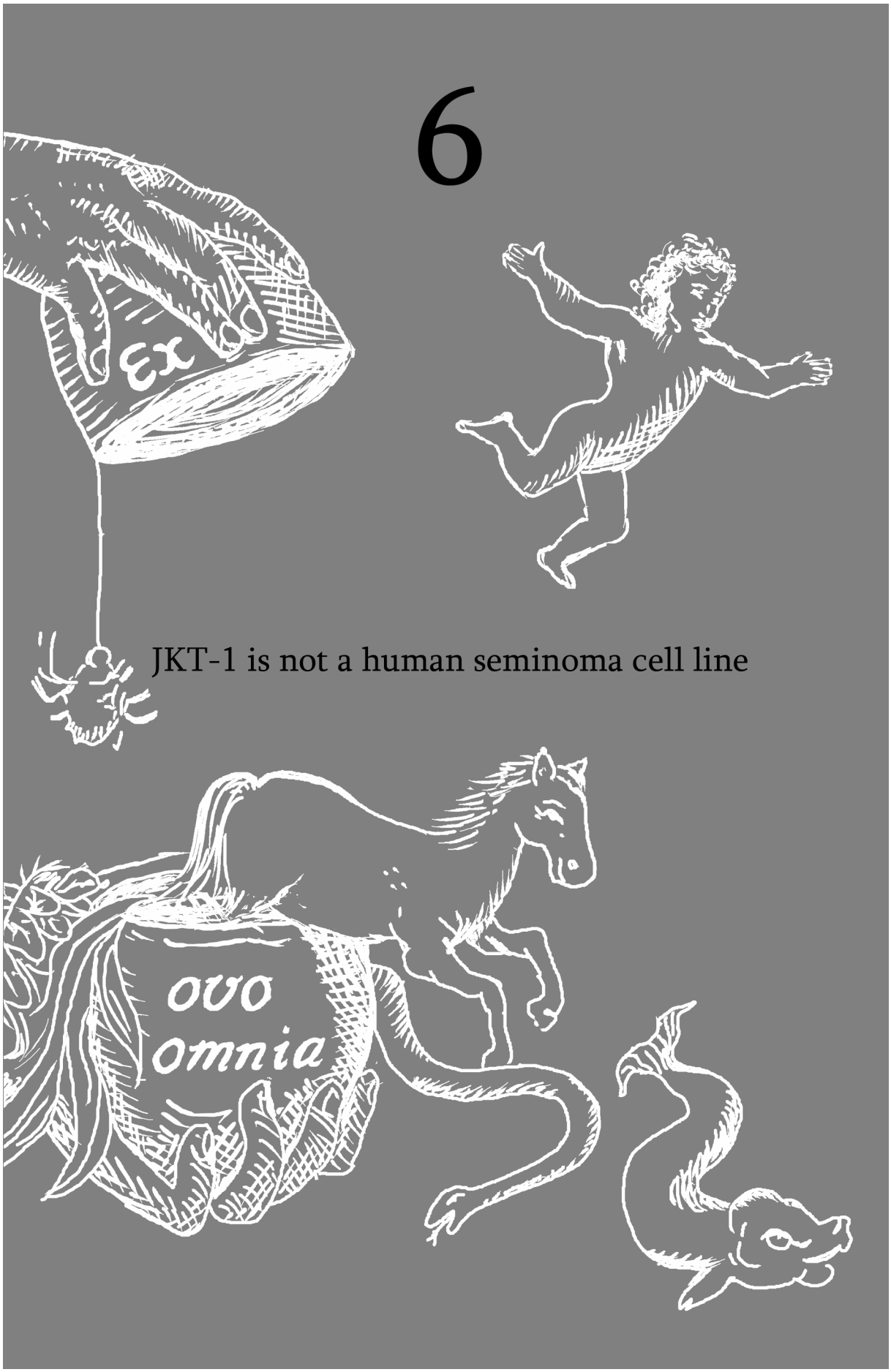

JKT-1 is not a human seminoma cell line

JKT-1 is not a human seminoma cell line

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ABSTRACT

The JKT-1 cell line has been used in multiple independent studies as a representative model of human testicular seminoma. However, no cell line for this specific tumour type has been independently confirmed previously; and therefore, the seminomatous origin of JKT-1 must be proven. The genetic constitution of the JKT-1 cells was determined using flow cytometry and spectral karyotyping, as well as array comparative genomic hybridization and fluorescent in situ hybridization. Marker profiling, predominantly based on differentially expressed proteins during normal germ cell development, was performed by immunohistochemistry and Western blot analyses. Moreover, genome wide affymetrix mRNA expression and profiling of 157 microRNAs was performed, and the status of genomic imprinting was determined. A germ cell origin of the JKT-1 cells was in line with genomic imprinting status and marker profile (including positive staining for several cancer-testis antigens). However, the supposed primary tumour, from which the cell line was derived, being indeed a classical seminoma, was molecularly proven not to be the origin of the cell line. The characteristic chromosomal anomalies of seminoma, e.g. gain of the short arm of chromosome 12, as well as the informative marker profile (positive staining for OCT3⁄4, NANOG, among others) were absent in the various JKT-1 cell lines investigated, irrespective of where the cells were cultured. All results indicate that the JKT-1 cell line is not representative of human seminoma. Although it can originate from an early germ cell, a non-germ cell derivation cannot be excluded.

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INTRODUCTION

Generation of cell lines derived from cancer cells of different organisms has been successful for more than half a century. Availability of these cell lines has been proven informative in various studies, although their impact on understanding the actual in vivo situation remains a matter of debate. Moreover, and at least equally important, the origin and purity of the cell lines currently used have been questioned, for relevant and obvious reasons. Proper molecular characterization and classification of cell lines will prevent scientific misinterpretation (Masters et al., 2001; Drexler et al., 2003). Besides verifying the origin and excluding contamination, it must be kept in mind that cells may undergo changes in their genome, epigenome and phenotype upon extensive and continuous in vitro culture because
of the non-physiological environment (Looijenga et al., 1997). This may hamper interpretation of the data obtained in various laboratories depending on specific culture conditions, even when cell lines of the same origin are analyzed.
Human germ cell tumours (GCTs) are a defined group of solid tumours, which can be classified based on various parameters, including histology and, to a certain level, chromosomal constitution (Oosterhuis & Looijenga, 2005). From a developmental point of view, the seminomas (SE) and non-SE, the so-called type II GCTs are of great interest. In the testis, these tumours (then called TGCTs) originate from a transformed embryonic germ cell, either a primordial germ cell (PGC) or gonocyte. This precursor lesion is referred to as carcinoma in situ (CIS) or intratubular germ cell neoplasia unclassified (Elbe et al., 2004; Skakkebaek, 1972). The invasive tumours are subdivided into seminomatous (SE) and non-seminomatous (NS) tumours. Subsequently, the NS can be subdivided into various subtypes: embryonal carcinoma (EC), teratoma, yolk sac tumour and choriocarcinoma, representing both undifferentiated and differentiated embryonic and extra-embryonic elements. In 1975, the first type II-GCTderived cell line was established by culturing EC (Fogh & Trempe, 1975). Since then, a series of NS-derived cell lines were successfully generated, used in various studies (Fogh, 1978). These have been very informative, although differences between various sub-lines, cultured in different laboratories, have been reported, for example regarding their pattern of X-inactivation (Looijenga et al., 1997). In contrast to the wealth of independently generated NS-derived cell lines, only single reports on cell lines representative for either CIS or seminoma are available. This is most likely because of the exceptional sensitivity of seminoma cells to undergo programmed cell death (apoptosis) upon disruption of their microenvironment, also known as anoikis (Berends et al., 1991; Olie et al., 1996). These observations might be related to the notion that PGCs rapidly go into apoptosis when they are situated at extragonadal locations during normal embryonic development (Stallock et al., 2003). Besides the type II TGCTs, a different type of GCT can occur in the testis. This so-called spermatocytic seminoma (type III) is rare, and can be distinguished from seminoma based on clinical behaviour, marker expression profile and chromosomal constitution (Eble, 1994; Rajpert-De Meyts et al., 2003b; Looijenga et al., 2006). No representative cell line for spermatocytic seminoma has been reported so far. Most recently, SE and spermatocytic

SE could also be distinguished from each other using microRNA (miRNA) expression profiling (Voorhoeve et al., 2006, and unpublished observations).
Interestingly, in 1993 Mizuno et al. reported on the establishment of a seminoma cell line, named TCam-2 (Mizuno et al., 1993). Up-to-date, the seminomatous origin of this cell line and retention of the characteristics have not been independently confirmed. A few years later, the establishment of the JKT-1 cell line was described (Kinugawa et al., 1998). It was derived from a testicular seminoma of a 40-year-old Japanese male. By (immuno)histopathological criteria, the primary tumour was a pure seminoma, positive for placental-like alkaline phosphatase (PLAP) and negative for alpha fetoprotein (AFP) and beta human choronic gonadotropin (β-hCG). These last two are markers for yolk sac and trophoblastic differentiation, respectively. The cell line was generated using the traditional explant culture method and the tumour cells were successfully transplanted into nude mice. Subsequently, a highly metastatic sub-cell line, named JKT-HM, was derived (Jo et al., 1999). A worrying finding is that the chromosomal analysis of both the original and the metastatic variant of JKT-1 lacked the characteristic chromosomal anomalies for seminoma, including gain of the short arm of chromosome 12, a genomic hallmark for invasive type II GCTs (Looijenga et al., 2003c). In fact, overrepresentation of 12p sequences is present in all invasive SE and NS, independent of histological composition of the latter, in up to 80% because of the presence of one or more isochromosomes 12p [i(12p10)] (Atkin & Baker, 1982; Looijenga et al., 2003c). This has been successfully used for diagnostic purposes (Bosl et al., 1994).
So far, the JKT-1 cell line has been used for various investigations, consistently described as representative of seminoma (Fujii et al., 2002; Hatakeyama et al., 2004; Kobayashi et al., 2004; Roger et al., 2004, 2005; Shiraishi et al., 2005) and the data obtained from the different studies were subsequently interpreted as such. Because of the uniqueness of a cell line representative of seminoma and the impact of its existence, both clinically and (patho-)biologically, we undertook an extensive characterization of the JKT-1 cell line, from genomic constitution to immunohistochemical marker profile. JKT-1 cell lines maintained in independent laboratories were included in the survey, as well as the (supposed) tumour of origin (Kinugawa et al., 1998).

MATERIALS AND METHODS

Cell lines and primary tumour

JKT-1 cells were obtained twice independently from Michiko Fukuda (The Burnham Institute, La Jolla, CA, USA), designated JKT-1 I and II, and Chris Lau (VA Medical Center, University of California, San Francisco, CA, USA), named JKT-1 III. The cells were cultured in a-modified Eagle medium + GlutaMAX (32564-029, Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum and penicillin/streptomycin in a 5% CO2 humidified incubator and passaged when 80% confluency was reached. Cells were washed twice with PBS and then incubated with Trypsin-EDTA (25300-054, Invitrogen) and harvested for the different experiments. Cytospins were prepared and

DNA, RNA and protein were isolated (see below). All sub cell lines were analysed by immunohistochemistry. Only JKT-1 I was used for expression profiling, determining of imprinting status, as well as mutation- and chromosomal analyses.
Paraffin-embedded specimens of the primary tumour were provided by Shingo Hatakeyama.

Immunohistochemistry

Immunohistochemical staining was performed on cytospin preparations of the JKT-1 cells I-III and on 3 µm thick tissue sections of the paraffin-embedded primary tumour. General markers as vimentin and cytokeratin 8 (CAM5.2), and specific markers for various maturation stages of germ cell development were applied. Published data on the expression of the markers PLAP, OCT3/4, NANOG, c-KIT, TSPY, DAZ, DAZL, VASA, MAGE-A4, DMRT1, SSX2-4, XPA, SYCP1 and SYCP3 in spermatogenesis and germ cell tumours is collected in Table 1 (Lammers et al., 1994; Lau et al., 2000; Reijo et al., 2000; Stoop et al., 2001; Lifschitz- Mercer et al., 2002; Zeeman et al., 2002; Looijenga et al., 2003b, 2006; Rajpert-De Meyts et al., 2003a; Hoei-Hansen et al., 2005).

TABLE 1. Immunohistochemical profile of consecutive maturation stages of male germ cells

	Spermatogenesis					Germ cell tumours		JKT-1			
Markers	PGC/ gonocyte	Prespermato- gonium	Spermato- gonium	Spermatocyte	Spermatid	Seminoma	Spermatocytic seminoma	Primary tumour	I	II	III
Positive in spermatogenesis											
PLAP	+	+/-	-	-	-	+	-	+	-	-	-
OCT3/4	+	+/-	-	-	-	+	-	+	-[c]	-[c]	-[c]
NANOG	+	-	-	-	-	+	-	+	-[c]	-[c]	-[c]
c-KIT	+	+	-	-	-	+	-	+	-[e]	-[e]	-[e]
TSPY	+/-	+	+	+/-	-	+/-[a]	+[a]	-	-[d]	-[d]	-[d]
DAZ	+	+	+	+/-	+/-	+/-	+	-	-[d]	-[d]	-[d]
DAZL	+	+	+	+	+	+/-[a]	+/-[a]	-	+/-	+/-	+/-
VASA	+/-	+/-	+/-	+	+	+/-[a]	+	-	+/-	+/-	+/-
MAGE-A4	-	+	+	+/-	-	+/-[a]	+[a]	-	-	-	-
DMRT1	nd	-[b]	+[b]	+[b]	-[b]	+/-	+	-	+	+	+
SSX2-4	nd	nd	+/-	+/-	-	+/-	+	-	-	-	-
XPA	-	-	-	+	-	+/-	+	-	+[c]	+[c]	+[c]
SYCP1	nd	-	-	+	-	-	+	nd	+[c]	+[c]	+[c]
SYCP3	nd	-	-	+	-	+[b]	+[b]	nd	+[c]	+[c]	+[c]
Negative in spermatogenesis											
CAM5.2								-	+	+	+
Vimentin								+/-	+	+	+
AFP								-	-	-	-
hCG								-	-	-	-

Spermatogenic cells (left panel: from left to right); germ cell tumours: seminoma and spermatocytic seminoma (middle panel); and cell line JKT-1 as well as (supposed) matched primary tumour (right panel). Positive staining in >90% of cells (+), weak or heterogeneous staining (±), negative (-) staining in >95% of cells; not determined (nd). PLAP, placental-like alkaline phosphatase; CAM5.2, cytokeratin; AFP, alpha fetoprotein; hCG, human chorionic gonadotropin. [a]Unpublished results from tissue microarrays (TMA) including 50 primary seminomas and 23 spermatocytic seminomas (Looijenga et al., 2006) are indicated; [b]own observations: protein expression of DMRT1 in spermatogenesis; [c]confirmed by Western blotting; [d]confirmed by PCR; [e]confirmed by immunoprecipitation and Western blotting.

In addition, the samples were stained for CD34 and CD45 to identify haematopoietic cells and inhibin-a for sertoli cells. The primary antibodies, dilutions and incubation conditions are listed in Table 2. For cytospin preparations, the same dilutions were used as described for the tissue sections with a standard 1-h incubation at room temperature (RT). On paraffin sections heatinduced antigen retrieval (HIAR) was performed by high pressure-cooking using 0.01 M sodium citrate (pH 6.0) for most antibodies. The antibodies directed against AFP and β-hCG required no antigen retrieval; for CAM5.2 staining, slides were treated for 15 min with protease (P5147; Sigma, Steinheim, Germany) 0.01% in PBS at 37°C, and HIAR for DMRT1 was performed in 1.0 M Tris–EGTA (pH 8.0). Secondary biotinylated antibodies SWine-Anti-Rabbit, rabbit-anti-mouse (DAKO, Glostrup, Denmark) and Horse-Anti-Goat (HAG) (Vector Laboratories, Burlingame, CA, USA) were used in a 1:200 dilution and incubated for 30 min at RT. Antibody complex was visualized by avidin-biotin conjugated with horseradish peroxidase or alkaline phosphatase, respectively using 3,3-diaminobenzidine and New Fuchsin (72200; Fluka, Buchs SG, Switzerland) as chromogens, resulting in a brown and a red signal. All slides were counterstained with haematoxylin. In addition, double staining was performed for DAZL and VASA, respectively, with 3-amino-9-ethyl-carbazole (A5754; Sigma)/H2O2 for a red staining and Fast Blue / Naphthol AS-MX phosphate (F3378 and N500; Sigma) for a blue staining.

TABLE 2. Origin and conditions used for primary antibodies

	Origin	Dilution	Incubation for paraffin
AFP	A0008, DAKO, Glostrup, Denmark	1:600	Overnight, 4°C
AR	(Zegers et al., 1991)	1:50	½h, RT
CAM5.2	345779, BD Biosciences, San Jose, CA, USA	1:20	2h, RT
c-KIT	A4502, DAKO	1:100	Overnight, 4°C
CD30	M751, DAKO	1:100	Overnight, 4°C
CD34	MS-363-P, Neomarkers, Fremont, CA, USA	1:30	½h, RT
CD45	M701, DAKO	1:200	½h, RT
DAZ	Provided by F. Moore and R. Reijo Pera (Reijo et al., 2000)	1:400	2h, RT
DAZL	Ab17224, Abcam, Cambridge, UK	1:750	2h, RT
DMRT1	Provided by D. Zarkower and V. Bardwell (Raymond et al., 2000)	1:400	2h, RT
HCG	A231, DAKO	1:10000	Overnight, 4°C
Inhibin α	MCA951S, Serotec, Oxford, UK	1:50	Overnight, 4°C
LHR	Provided by A. Themmen; (Funaro et al., 2003)	1:6400	Overnight, 4°C
MAGE-A4	Provided by H. Jacobs; (Kocher et al., 1995)	1:100	2h, RT
NANOG	AF1997, R&D Systems, Abingdon, UK	1:800	2h, RT
OCT3/4	sc-5279, Santa Cruz Biotechnology CA, USA	1:1000	2h, RT
P53	M7001, DAKO	1:50	2h, RT
PLAP	203, Cell Marque Corp, Hot Springs, AR, USA	1:200	Overnight, 4°C
SYCP1	Provided by C. Heyting (Meuwissen et al., 1992)	1:500	2h, RT
SYCP3	Provided by C. Heyting (Lammers et al., 1994)	1:500	2h, RT
SSX2-4	Provided by A. Geurts van Kessel (dos Santos et al., 2000)	1:100	2h, RT
TSPY	Provided by Y.-F. C. Lau (Lau et al., 2000)	1:4000	Overnight, 4°C
VASA	Provided by D. Castrillon (Castrillon et al., 2000)	1:1000	Overnight, 4°C
Vimentin	M725, DAKO	1:80	2h, RT
XPA	MS-650-P, Neomarkers	1:50	1h, RT

Protein isolation and Western blotting

Isolation of protein and Western blotting were performed as described previously (de Jong et al., 2005).

Genotyping

Allelotyping was performed by radioactive polymerase chain reaction (PCR)-amplification of five highly polymorphic microsatellite markers: D9S156, D9S157, D17S786, D8S133 and D8S136 using standard methods.

Flow cytometry

Cellular DNA content and cell cycle characteristics were determined using a FACS scan flow cytometer (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA). In total 10 000 events were analyzed. Briefly, JKT-1 cells were collected and fixed with cold 70% ethanol. Cells were resuspended in 1 ml of PBS containing propidiumiodide (50 lg / ml), glucose (1 mg / ml) and RNase A (0.1 mg / ml). The suspensions were incubated at RT for 30 min. The ploidy determination of nuclei was estimated by flow cytometry of DNA content in relation to an independent proven control with a diploid DNA content.

Spectral karyotyping

Standard cytogenetical preparations were pre-treated with RNase for 30 min at 37°C and digested in 0.012% pepsin for 5 min. After washing, cells were fixed in 1% formaldehyde / 50-mM MgCl2 in PBS for 10 min, washed, and dehydrated. Hybridization and detection for Spectral karyotyping (SKY) analysis was performed according to the manufacturer's protocol with minor adjustments (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Briefly, after 2 days of hybridization, slides were washed in 4xSSC (pH 7.0) for 15 sec at 72°C followed by 2xSSC at RT. Cells were counterstained with DAPI and mounted in antifade solution (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, CA, USA). Using the SD300-C SpectraCube and Skyview analysis software (Applied Spectral Imaging), nine metaphase cells were examined (Naus et al., 2001).

DNA isolation

Genomic DNA from the primary tumour and the cultured cells was extracted as described before (Kersemaekers et al., 2002). Briefly, each sample was treated with proteinase K followed by phenol / chloroform extraction and DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

Array-comparative genomic hybridization

A human BAC/PAC array containing 3600 clones covering the full genome at an average of 1 Mb-spacing, was used (Cardoso et al., 2004). Labelling and hybridization were performed as described before (Cardoso et al., 2004). Scanning was performed on a ScanArray Express HT Microarray Scanner (Perkin-Elmer, Fremont, CA, USA) and the resulting images were analyzed with the GenePix Pro 5.0 software (Molecular Devices Corp., Sunnyvale, CA, USA). Breakpoint flanking clone positions were verified with Ensemble (v36, December 2005; http://www.ensemble.org). The baseline DNA copy number (log2=0) in the JKT-1 cell line referred to triploidy.

Fluorescent in situ hybridization

FISH was performed on cytospins of JKT-1 cells and sections of the primary tumour as described before (Zafarana et al., 2002). The following probes were used: RP11-344A7 (9p21.3), RP11-621O18 (9p22.3), RP11-117 J28 (9p23), RP11-28O4 (9q32.33), RP11-417B4 (9q33), RP11-83 N9 (9q34.13), RP11-277 J24 (12p13.1 containing NANOG), RP11-449P1 (12p12.1) and RP11-268P4 (12p12.1) [the latter two clones containing EKI1, a gene located at the telomeric region of the shortest region of overlap of amplification in SE: p11.2-p12.1 (Zafarana et al., 2002)], TSPY plasmid of 12.5 kb (Zhang et al., 1992) and centromere-specific probes D9Z1 (Moyzis et al., 1987), Pα12H8 (Looijenga et al., 1990), DXZ1 (Willard et al., 1983) and DYZ3 (Cooke et al., 1982), respectively for chromosome 9, 12, X and Y. Centromere specific probes were labelled with biotin-16-dUTP (Roche, Mannheim, Germany) and detected using streptavidin-Cy3 (Jackson Immuno Research Laboratories, Cambridgeshire, UK), the other probes were labelled with digoxigenin-11-dUTP (Roche) and visualized with FITC-conjugated sheep anti-digoxigenin (Roche).

Multiplex PCR AZF regions

Screening for micro-deletions of the Y chromosome was performed by a standardized multiplex PCR as described in Simoni et al. (2004). The STS primers used were sY84 (DYS273) and sY86 (DYS148) for the AZFa region, sY127 (DYS218) and sY134 (DYS224) for AZFb and sY254 (G38349) and sY255 (G65827) for AZFc, both primers were located in the DAZ gene. As controls, the SRY gene on Yp, normal male and female DNA were taken, and the ZFX / ZFY gene served as an internal control. This primerset enables the detection of almost all the clinically relevant deletions (resulting in azoospermia or oligospermia) and over 95% of the deletions reported in the literature in the three AZF regions (Simoni et al., 2004).

Imprinting status of IGF2/H19 imprinting control region

To determine whether imprinted genes are either monoallelically or bi-allelically expressed in the JKT-1 cells, the ApaI and RsaI polymorphisms within the IGF2 / H19 imprinting control region (ICR) were investigated. Therefore, DNA of cultured cells was amplified at both loci by PCR, followed by restriction enzyme digestion. In case heterozygosity was determined, a similar analysis was performed on cDNA (van Gurp et

al., 1994). The following primers were used: 5'-CTTGGACTTTGAGTCAAATTGG-3' and 5'-GGGTCGTGCCAATTA-CATTTCAT-3' (IGF2 locus); 5'-TGACTGAGGAATCGGCT-CTGGAAG-3' and 5'-CGGTCG-GAGCTTCCAGACTAG-3' (H19 locus).

Methylation status

IGF2/H19 ICR

The methylation status of the *IGF2/H19* ICR was determined by methylation-sensitive single nucleotide primer extension (MS-SNuPE), PCR and sequencing of the CTCF core binding domain as described by Sievers et al. (2005). The SNuPE primer sequence for the first cytosine in the CTCF binding domain was C_{10}-GTTGTGGAAT(C/T)GGAAGTGGT (primer A) and for the first cytosine before the CTCF core domain was C_{12}-GAATTGGTTGTAGTTGTGGAAT (primer B). Primers for amplifying the CTCF core domain were 5'-TGTATAGTATATGGGTATTTTTGGAGGTTT-3' and 3'-GGGAAT-AGGATATTTA-TAGGA-5'. Sperm DNA was used as a control.

OCT3/4 promoter

Bisulfite treatment was carried out on isolated DNA (see above), using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) as described before (de Jong et al., 2007). Amplification was performed by means of PCR using the primer pairs OCT4-3 to OCT4-9 as described by Deb-Rinker et al. (2005). PCR products were sequenced using radioactive labelling.

Expression analysis

Microarray expression profiling

Expression profile of the JKT-1 cell line was determined using Affymetrix GeneChip Human Genome U133 plus 2.0 array as described before (Looijenga et al., 2006). Previously published expression data of patient derived GCT samples were used for clustering (Looijenga et al., 2006).

MicroRNA expression profiling

RNA highly enriched for low molecular weight (LMW) RNA species (LMW) was isolated using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA quantity and quality were determined using a high-resolution electrophoresis bioanalyser 2100 (Agilent, Amstelveen, NL) and by UV absorbance.

Expression of 157 mature miRNAs was analyzed by using TaqMan miRNA Assays Human Panel (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Briefly, single-stranded cDNA was synthesized from 10 ng of LMW RNA, using the so-called hair-pin RTprimer, in 15 µl reaction volume by using the TaqMan miRNA Reverse Transcription Kit. The reactions were incubated first at 16°C for 30 min and then at 42°C for 30 min. The reactions were inactivated by incubation at 85°C. Each cDNA generated was amplified by Quantitative PCR (Q-PCR) using sequence-specific primers/probes on a fully automated Applied Biosystems 7900HT Real-Time PCR system. The 10 µl PCR

included 5 µl Universal PCR Master Mix (Applied Biosystems; No AmpErase UNG), 1 µl of sequence-specific primers/probes and 1.5 µl of RT-product. The reactions were incubated in duplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Mir-16 and let-7 were used as internal controls. The median threshold value of the controls was determined and the difference with the target threshold was determined as delta CT. This was used to investigate the different samples for expression of the targets along all samples included in the survey. Using the Omniviz tools, biostatistical analyses were performed on the total data set as described before (Looijenga et al., 2006).
In addition, the presence of miRNA-372 and 373 in SE, was determined as described recently by Voorhoeve et al. (2006).

Mutation analysis of *P53, KRAS2* and *BRAF*

To investigate the presence of *P53* mutations, in this case in exon 5–8, the single-strand conformation polymorphism approach as described before (Kersemaekers et al., 2002) was applied. If aberrant bands after gel electrophoreses were identified, these were sequenced.
KRAS2 and *BRAF* mutation analysis was performed by locked nucleic acid-mediated PCR clamping and melting curve analysis followed by sequencing. For *KRAS2* primers, oligomer and probe were used from Chen et al. (Chen et al., 2004). Oligonucleotides used for *BRAF* mutation analysis were the following: CATCTCAGGGCCAAAAAT (BRAF-F), TCCTTTACTTACTACACCTCAG (BRAF-R), GAGATTTCACTGTAGCTAGACCAA-Flu (sensor), LC Red 640-TCACCTATTTTTACTGTGAGGTCTTCATGAAGAAA-Ph (anchor), GAGATTT-CTCTGTAGCTA-Ph (LNA).

RESULTS

Morphology and immunohistochemistry of the primary tumour and JKT-1 cells

The primary tumour from which the JKT-1 cell line was reported to derive consists of sheets of uniform cells with clear cytoplasm and large nuclei mingled with lymphocytic infiltrations. Immunohistochemistry of the tumour cells confirmed the morphological diagnosis of a seminoma, with a consistent strong nuclear positive staining for both OCT3/4 (Fig. 1A) and NANOG, a predominantly membranous staining for PLAP and c-KIT (Oosterhuis & Looijenga, 2005; Table 1). All markers included in this study (Tables 1 and 2) specific for later stages of spermatogenesis, which are positive in spermatocytic seminoma, like DMRT1, SSX2-4 and XPA were negative in the primary tumour (Table 1), consistent with the seminoma histology. Markers reported previously to be heterogeneously expressed in SE, like MAGE-A4, DAZL and TSPY (Table 1), were negative in the primary tumour. Based on the immunohistochemical data and morphology, the primary tumour is a classical seminoma.

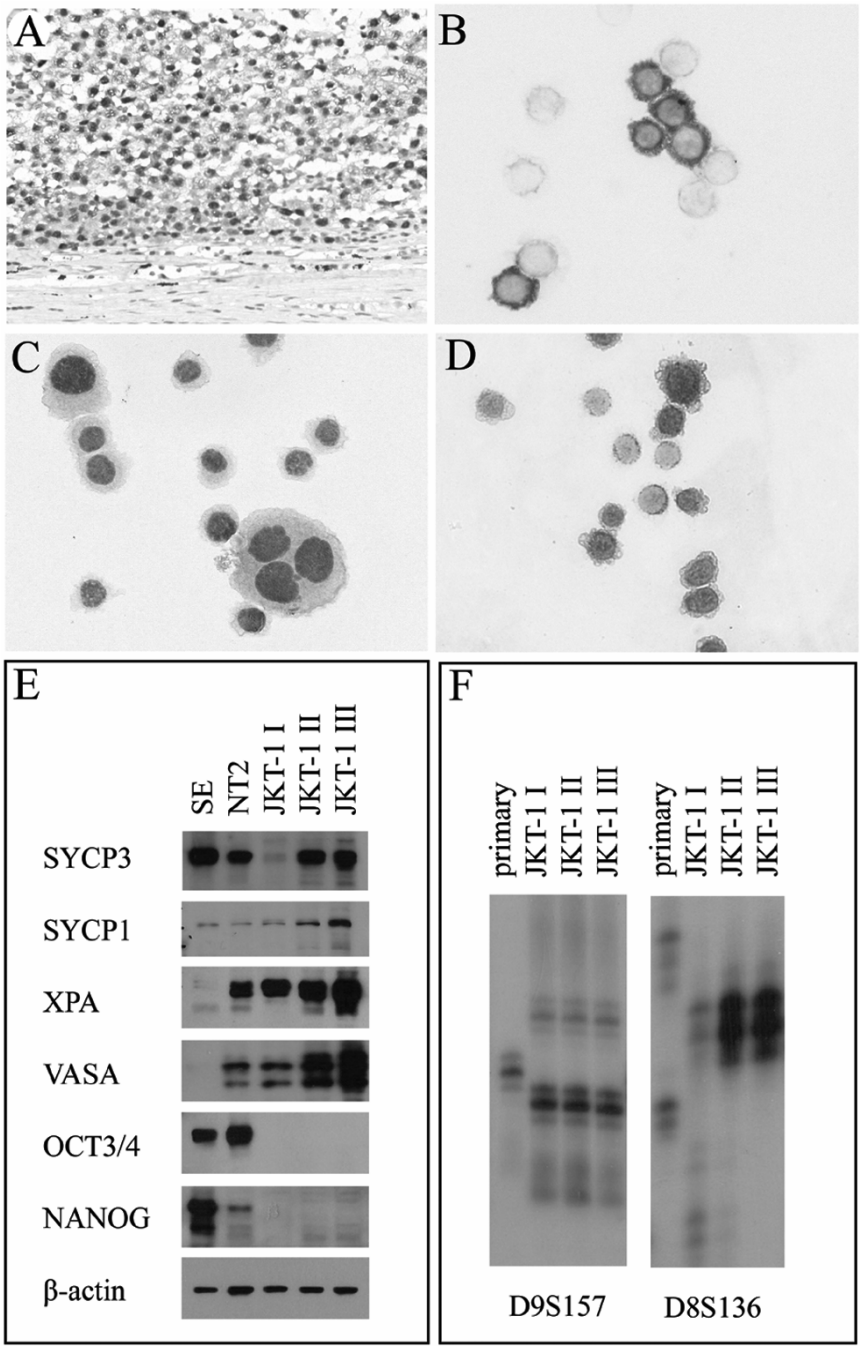


FIGURE 1.
(A) Immunohistochemistry of the primary tumour for OCT3 / 4;..... immunohisto-chemistry of JKT-1 cells: (B) double staining for VASA (red color) and DAZL (blue color). Note the more intensive DAZL staining in the cells positive for VASA; (C) XPA; (D) SYCP3; (E) Western blot analysis of a seminoma, the EC-derived cell line NT2, and the three subclones of the JKT-1 cell lines (I, II, and III), for SYCP3, SYCP1, XPA, OCT3 / 4, NANOG and VASA, ß-actin was used as a loading control; (F) clonality analysis of the supposed primary tumour and the three JKT-1 cell lines, designated I, II and III. The following markers were applied: D9S156, D9S157, D17S786, D8S133, and D8S136. Note that the different sub-lines show the same pattern, meaning a monoclonal origin, but these were clonally unrelated to the primary tumour. The limited number of bands per marker indicate that both cell lines and primary tumour have no micro-satellite instability. *(Figure 1 colour image on page 182)*

JKT-1 cells grown in vitro formed colonies of polygonal cells, with polynuclear cells in up to 5%. Up to 60% of the cells were positive for VASA, both with immunohistochemistry and Western blotting (Fig. 1B and 1E). In addition to VASA, these cells were positive for DAZL (Fig. 1B). In contrast to the primary tumour, the JKT-1 cells were positive for a series of markers found to be specific for later stages of normal germ cell development, including DMRT1, XPA (Fig. 1C and E) and meiosis markers SYCP1 and SYCP3 (Fig. 1D and 1E), both by immunohistochemistry and Western blotting. The cells were consistently negative for OCT3/ 4 (Fig. 1E), NANOG (Fig. 1E), enzymatic alkaline phosphatase, PLAP (<1% positive), and c-KIT (Table 1). The JKT-1 cells were also positive for vimentin and CAM5.2, while others, like CD30 and CD45 were negative. No

difference in staining pattern was identified for the three sub-lines (I, II and III) included in this survey (Table 1). Therefore, they will not be referred separately in this report, except when relevant.

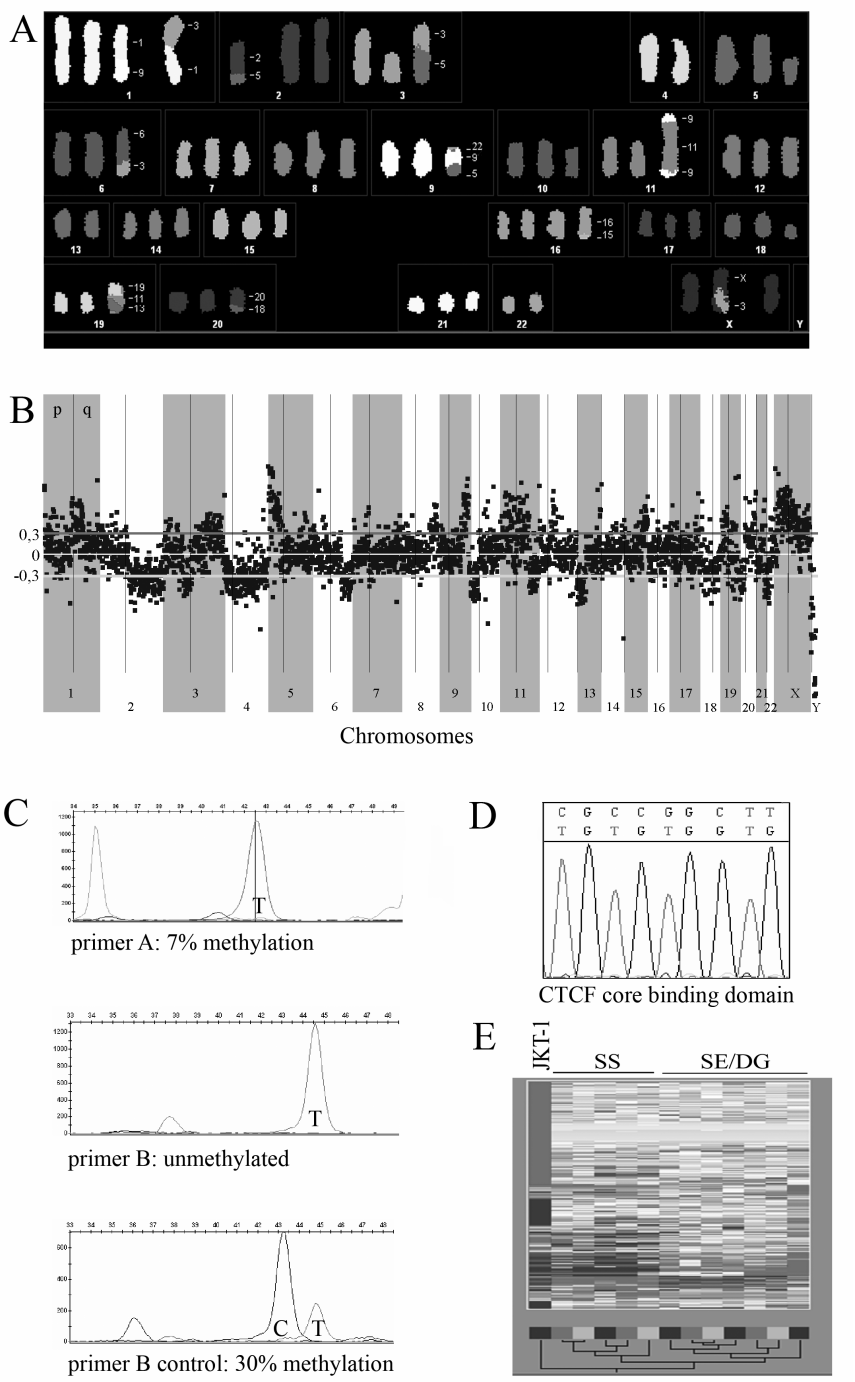
Primary tumour and JKT-1 cell line are not related

Because of the striking differences between the characteristics of the primary tumour and the supposedly derived cell lines, we investigated their clonality using multiple informative molecular markers. These markers are used in daily clinical practice to identify possible monoclonal origin of multiple tumours in a single patient, and mix-up of patient samples. The results clearly demonstrate that the three sub-lines of JKT-1 obtained from the different laboratories are monoclonal and of human origin (Fig. 1F), in line with their similar marker expression (Table 1). Moreover, it indicated that the marker profile of the cell line grown in vitro is rather stable and has no micro-satellite instability. In contrast, the primary tumour is unrelated to the cells grown in culture (Fig. 1F). All markers applied showed discordance between the cell lines and primary tumour, revealing that the JKT-1 cell line is not derived from this tumour. Despite multiple attempts, the cell line could not be traced back to its origin. Therefore, the seminomatous histology of the supposed primary tumour cannot be used as supportive evidence that the cell line is representative for seminoma. Based on these findings, the primary tumour was not included for further investigation in this study.

Genomic constitution of the JKT-1 cells

The ploidy and growth characteristics of the JKT-1 cells were determined by flow cytometry. In accordance with the original description of the cell line, the cells show a triploid DNA content (index of 1.4), with a distribution in G1 and G2-M-phase respectively of 43 and 33% (data not shown). The near triploid status was confirmed by SKY, showing that metaphase spreads with the number of chromosomes ranging from 61 to 68. The most prominent genomic aberrations were loss of chromosomes 4, 13, 22 and Y, and gain of 5p (present as an isochromosome) and X. A representative metaphase analysed by SKY is shown in Fig. 2A (of which the consensus constitution is indicated in the legend). Array CGH (Fig. 2B) showed loss of fragments of 2q, the centromeric region of chromosome 3, the telomeric region of 6q, 10p, centromeric region of 13 and chromosomes 4, 18, 22 and Y. Gain was found for 5p, 9q and chromosome X. In contrast, no gain of 12p was identified in either of the metaphase spreads investigated and confirmed by FISH with region-specific probes. The findings in SKY and array-CGH are overall consistent with each other. For example, chromosome 4, 13 and 22 are found in two copies, and show loss in the array CGH approach. Accordingly, chromosomes 7, 8, 12, 14, 15, 17 and 21 are present in three copies and balanced in the array CGH profile. Chromosome 9 was investigated in more detail because of the recurrent gains in

FIGURE 2



(A) Representative metaphase of spectral karyotyping analysis of JKT-1. Characteristic aberrations are loss of chromo-somes 4, 13, 18, 22 and Y; gain of X; and isochromosome 5p. The clonal chromosomal constitution based on nine metaphases is: 61-68,XX,+der(X)t(X;3)(q22;?) [7] -Y,der(1) t(1;9)(q21;q?)[8],der(1;3) (q10;p10)[8], der(2)t(2;3)(q21;q2) [2],der(2)t(2;5)(q21;?),der(3)del(3) (p?) del(3) (q?) [2], der(3;5), (p10; q10)[8],-4,+i(5)(p10), der(6) t(3;6) (?;q22)[7],del(7)(p21)[3],-9,add(9) (p11) [4], -10[3], del(10)(p1) [2], der(10)t(3;10)(?;p1?2)[6], der(11) dup(11) (p?p?) t(3;11) (?;p14) dup(11) (q?q?) t(3;11) (?;q24)[3], der(11)dup(11)(p?p?)t(9;11)(?;p14) dup(11)(q?q?)t(9;11)(?;q24)[6],-13, +der(16) t(15;16) (?;q23), -18[7], der(18) -del(18) (p1) del(18) (q?1)[2], der(19) (19::11::13) [8], der(19)t(19;20)(?;?)[2],der(20)t(18; 20)(?;?)[5],-22, +der(?) (5::9::X) [8], +der(?)(5::9::X)[2], +der(?) (?;3)[2] [cp9]. (B) Results of the 3.6 K BAC / PAC array CGH analysis of the JKT-1 cells. See Table 3 for detailed description; (C) methyl-ation-sensitive single nucleotide primer extension (MS-SNuPE) of the IGF2 / H19 imprinting control region of JKT-1 shows a very low methylation level by two independent primers (7% for primer A and 0% for primer B). In contrast, the sperm DNA control has a 30% methylation level; (D) Sequencing of the CTCF core binding domain on bisulfite treated DNA of JKT-1 confirms the MS-SNuPE data. All cytosines (initial sequence on top) have been converted to thymine because they were unmethylated (bisulfate treated DNA sequence below); (E). Results of Affymetrix expression profiling. Unsupervised clustering of JKT-1, 5 spermatocytic seminomas (SS), 4 seminomas (SE) and 3 dysgerminomas (DG) using Omniviz. The SE and DG could not be separated, in contrast to the SE/DG and SS. JKT-1 does not cluster with any of the tumour samples. *(Figure 2 colour image on page 183)*

spermatocytic SE (Looijenga et al., 2006), and the multiple breakpoints affecting chromosome 9 in the JKT-1 cells (SKY results). This is of specific interest because of the positive staining of the JKT-1 cells for DMRT1 (which maps to 9p21) that was identified recently as a candidate gene in the genesis of spermatocytic seminomas (SS) (Looijenga et al., 2006). In fact, chromosome 9 sequences were found in association with chromosome 1 and 11 in the SKY analysis. However, array CGH did not indicate that (part of) chromosome 9 was gained, with the exception of 9q33-9qtel, as confirmed by FISH (data not shown). The absence of the complete Y-chromosome detected in array-CGH and SKY analysis was supported by FISH and multiplex PCR for the AZF regions (data not shown). The results on array-CGH, SKY and FISH in comparision with previously obtained results (Jo et al., 1999) are summarized in Table 3.

TABLE 3. Comparison of genomic aberrations of JKT-1 as found by array CGH, SKY, FISH and CGH.

	Array CGH						
Chromosome	Gain/ Loss	Chromosome band	Flanking clones	Size (Mb)	SKY	FISH	CGH (Jo et al., 1999)
1	gain	p13.3-q25.3	RP11-28P8 - RP11-293B7	72			+1q
2	loss	q12.3-qter	RP11-443K8 - RP11-556H17	133			
3	loss	p14.2-q12.1	RP11-154D3 - RP11-569H14	37			
4	loss	chr. 4		191	-4		
5	gain	p12-pter	RP11-28I9-b - CTD-2265D9	42	i(5p)		
6	loss	q22.1-qter	CTB-57H24 - RP1-94G16	52			
7	loss	p11.2-q11.22	RP4-725G10 - RP11-409J21	14			
8	gain	q24.11-qter	RP11-67N21 - CTC-489D14	27			
9	gain	q33.1-qter	RP11-574M5 - GS1-135I17	18		3c,5q[a]	
10	loss	p11.21-pter	CTB-164I22 - CTC-306F7	37			
11	gain	pter-q22.2	CTC-908H22 - RP11-21G19	100			+11
	loss	q22.2-qter	RP11-305O6 - RP11469N6	32			
12	gain	q12-q13.3	RP11-283I17 - RP11-181L23	12		3p, 3c	
13	loss	q12.11-q14.3	RP11-76K19 - RP11-200F15	34	-13		
15	gain	q26.1-qter	RP11-405A15 - CTB-154P1	106			
17							+17q
18	loss	chr. 18		77	-18		-18p
20	loss	cen-pter	RP11-348I14 - CTB-106I1	29			
22	loss	cen-q12.3	XX-p8708 - CTA-221H1	17	-22		
X	gain	chr. X		154	XX	3X	+X
Y	loss	chr. Y		57	-Y	-Y	-Yq

To simplify the overall picture, only distinctive aberrations for SKY were included. CGH data were derived from published data (Jo et al., 1999). FISH column: number of copies centromere (c), p-arm (p) and q-arm (q). [a]With q probes in q33.1-qter.

Imprinting and methylation

The pattern of genomic imprinting established during normal germ cell development (Surani, 1998), has been used to characterize the origin of the seminomatous and NS GCTs as well as SS (van Gurp et al., 1994). Because of non-informativity of the reported polymorphisms within the *IGF2/H19* locus, no data on bi- or mono-allelic expression of these genes could be obtained. However, the methylation status of the CTCF binding domain within this locus could be studied, which is also informative for imprinting status (Sievers et al., 2005). Overall, a low level of methylation was found (7% for primer A and 0% for primer B) (Fig. 2C). This was also found for the CTCF core-binding domain regarding all four CpG sites investigated (Fig. 2D). The data are consistent with an immature germ cell (Kawakami et al., 2006).
An inverse relationship has been found between expression of *OCT3/4* and methylation of its promoter in Ecderived cell lines (Deb-Rinker et al., 2005). We confirmed this pattern in primary tumour samples and showed that the JKT-1 cells, which lack expression of OCT3/4, contain a high level of methylation (de Jong et al., 2007). A similar level of expression is found in SS, amongst other tumour types of non-germ cell origin, while SE show an almost unmethylated pattern (de Jong et al., 2007).

Expression pattern

Affymetrix expression array analysis showed that the JKT-1 cell line did not cluster in either the seminoma or spermatocytic seminoma group (Fig. 2E). In fact, it is not more closely related to either of these histological subtypes. The data have to be interpreted with caution because of the comparison between primary tumours (seminoma and spermatocytic seminoma) and a cell line. In addition, JKT-1 clustered separately when compared with a published data set of 175 carcinomas of various histologies and organ of origin (Su et al., 2001) (data not shown). The mRNA expression data of JKT-1 and the GCT samples used for clustering are available as supplemental data (see Supplementary material; Tables S1–S3).
We previously showed that SE and SS have different patterns of gene expression, both by RT-PCR profiling and by comparative expressed sequence hybridization (CESH) (Looijenga et al., 2006). Most recently, we demonstrated that the majority of SE, in contrast with SS, expresses specific miRNAs, i.e. miRNA 372-373, that are members of the hsa-miRNA 371-373 cluster on chromosome 19 (Voorhoeve et al., 2006). This is most likely related to the presence of wild type of P53 in these tumours. The JKT1 cells were negative for this set of miRNAs, just like all SS investigated, while a selection of cell lines derived from EC were positive (data not shown). Based on these findings, a general profile based on a panel of 157 miRNAs was determined by Q-PCR in the JKT-1 cell line. The different miRNAs included in the study were grouped into three classes based on their relative expression level compared with the normalized control: high expression (>2 SD), intermediate (–2 SD to 2 SD) and low (under)2 SD) (Table 4). Within the group of highly expressed miRNAs, no direct correlation with DNA copy numbers could be identified (data not shown).

TABLE 4. MicroRNA expression profile of JKT-1

High
let-7d, 16, *17-5p*, *19a*, *20*, 21, 23a, 27a, 29a, 30b, 30c, *92*, 99a,100,106a
Intermediate
let-7a, let-7g, let-7i, 15b, 23b, 27b, 29b, 29c, 30a-3p, 31, 98, 103, 186, 221, 301, 320
Low
lin-4, let-7e, 9, 9*, 10a, 15a, 17-3p, 26a, 26b, 28, 30e, 34a, 34b, 34c, 95, 96, 104, 105, 107, 122a, 124a, 124b, 125a, 125b, 126, 127, 128a, 128b, 129, 130a, 130b, 132, 133a, 133b, 134, 135a, 135b, 137, 138, 139, 140, 141, 142-3p, 142-5p, 144, 145, 146, 147, 148a, 149, 150, 151, 152, 154, 154*, 155, 159a, 181a, 181b, 181c, 182, 182*, 183, 184, 185, 187, 189, 190, 191, 193, 194, 195, 197, 198, 199a, 199a*, 199b, 199-s, 200a, 200b, 200c, 203, 204, 205, 210, 211, 213, 214, 215, 216, 218, 219, 220, 222, 223, 224, 296, 299, 302, 302b, 302b*, 302c, 302c*, 302d, 308, 323, 324-5p, 325, 326b, 328, 330, 331, 335, 337, 338, 339, 340, 342, 367, 368, 370, **371**, **372**, **373**, **373***, 374

Panel of 157 human miRNAs were divided in groups of high (more than 2SD), intermediate (-2SD to 2SD) and low (under –2SD) expression level. Members of known clusters are indicated in bold (Voorhoeve et al., 2006), underlined (Suh et al., 2004) and italic (O'Donnell et al., 2005).

Mutation screening

Overall, SE have a very low-mutation rate in the genes encoding receptor kinases (Bignell et al., 2006). In contrast, mutations can be found in the stem cell factor receptor *c-KIT* (Kemmer et al., 2004), in particular in bilateral tumours (Looijenga et al., 2003a). Moreover, some mutations have been identified in the proto-oncogenes *KRAS2, BRAF*, and very seldom in *P53* (Kersemaekers et al., 2002; McIntyre et al., 2005; Sommerer et al., 2005). No mutations in *c-KIT, KRAS2, BRAF* and *P53* were found in the JKT-1 cell line (data not shown).

DISCUSSION

After the first report on the human cell line JKT-1 as a representative model of testicular seminoma, this cell line has been used in multiple studies. These relate to the functional role of trophinin (Hatakeyama et al., 2004), connexin 43 (Roger et al., 2004) and estrogen (Roger et al., 2005) in the pathogenesis of TGCTs. In addition, it has been used in the identification of the mechanisms related to cisplatin sensitivity and especially resistance (Kobayashi et al., 2004). It is known that overall TGCTs, both seminomatous and NS tumours, with the exception of teratoma, are highly sensitive to cisplatin-based chemotherapy. An observation of interest, while treatment of other types of solid cancers might benefit of elucidation of the mechanisms involved (Masters & Koberle, 2003). Moreover, the cell line has been investigated regarding its response at the so-called hGABP/ E4TF1 site of the retinoblastoma gene (RB) promoter (Shiraishi et al., 2005). The data obtained, although of interest, were interpreted with the assumption that the JKT-1 cells are derived from a testicular seminoma, and could serve as model for this tumour type.

Initially, based on the absence of gain of 12psequences, which is a characteristic chromosomal anomaly of all SE, in combination with the absence of representative cell

lines for this tumour type, we questioned the origin of the JKT-1 cell line, and initiated the study of which the results are reported here.
As demonstrated in this comprehensive study, the JKT-1 cells obtained from different laboratories are clonal in origin, and stable regarding the analysed protein expression. However, the cell line was not derived from the supposed primary tumour as proven by genotyping and immunohistochemistry. This conclusion was further strengthened by the chromosomal constitution of the cell lines, lacking the characteristic overrepresentation of 12p of invasive TGCTs (Atkin & Baker, 1982; Looijenga et al., 2003c), in accordance with the original report (Jo et al., 1999). This indicates that the lack of gain of 12p-sequences is not the result of extensive in vitro culturing.
The obvious and relevant question is what the cell of origin of the cell line JKT-1 is. The immunohistochemical profile of JKT-1 corresponds largely to a more mature germ cell, like a spermatogonium or spermatocyte (Table 1). The presence of SYCP1 and SYCP3 strongly supports the origin of a cell undergoing (partial) meiosis (see below). We have made a similar observation in SS (Looijenga et al., 2006). In addition, the co-expression for DAZL and VASA in 60% of the cells relates to the stabilizing function of DAZL on translation of VASA (Reynolds et al., 2005) as seen in pre-meiotic germ cells. Our immunohistochemical results deviate remarkably from those of the original article, in which JKT-1 was described. The initial paper reported that the cell line is positive for PLAP, whereas our stainings show the exact opposite: <1% of JKT-1 cells are positive for this marker. Based on the finding that the cells are also negative for the enzymatic activity of this enzyme, it can be excluded that this negative result is because of the use of, for example, different antibodies. The three sub-lines investigated showed the same pattern marker expression. This makes it unlikely that the different pattern of marker profile is because of specific culture conditions, because the cell lines from the other institutes were cultured only in our facility for a very limited time. We hypothesized that the JKT-1 cell lines originate from a spermatocytic seminoma. This tumour is less frequent than seminoma, and can be misdiagnosed as a classical seminoma (Stoop et al., 2001). The SS have a specific pattern of marker expression, different from classical seminoma (Rajpert-De Meyts et al., 2003b). They are positive for cancer-testis antigens XPA, SSX2-4 and SYCP1, amongst others and more recently, others were added, like DMRT1 (Looijenga et al., 2006). Interestingly, most of these markers were indeed found to be consistently positive in the JKT-1 cells. These observations support the model that the JKT-1 cells do indeed originate from a spermatocytic seminoma. However, the chromosomal constitution of the cells is not in accordance with the expected pattern, although so far only a limited number of this type of tumours have been investigated. We and others showed that the SS contain different populations of tumour cells, showing a diploid, a tetraploid and a hypertetraploid DNA content (Looijenga et al., 1994; Verdorfer et al., 2004). This is in contrast to the triploid DNA content of the JKT-1 cells. Moreover, all SS investigated so far, using karyotyping, SKY, array CGH, and FISH showed specific overrepresentation of chromosome 9, and a specific amplification of the 9p21-p24 region in a single case. The *DMRT1* gene has been found by us to be the most likely candidate within this region, being overexpressed in all SS investigated so far (Looijenga et al., 2006). Although DMRT1 is indeed expressed in the JKT-1 cells, no gain of this

chromosomal region could be identified, not even using FISH with region-specific probes. In this context, the specific methylation pattern within the *IGF2/H19* ICR should be noted. Compared with all other biological subtypes of GCTs, SS show the highest degree of methylation at this site, indicating a gain of methylation related to germ cell maturation, while the methylation imprint is at least partially erased in the other tumours (Sievers et al., 2005). As we detected a low methylation level in JKT-1, this finding also argues against the origin from a spermatocytic seminoma.
Otherwise, the significant structural chromosomal changes detected by us stand in sharp contrast to the minimal changes found previously by Jo et al. (1999), who characterized JKT-1 and JKT-HM >5 years ago with karyotyping and conventional CGH. These differences might be because of the extended in vitro growth of these cells, possibly related to the selection or induced changes.
Based on these results, as well as the mRNA and miRNA expression profiling, it can be definitely concluded that the JKT-1 cells are not representative for (testicular) seminoma. Therefore, the reported data on the role of trophinin, connexin 43, estrogen, mechanisms of cisplatin-sensitivity and RB-related cell cycle control must be re-interpreted in the context of the pathogenesis of TGCTs. It remains to be established whether the JKT-1 cell line is derived from a spermatocytic seminoma, or a normal spermatocyte. Alternatively, it might originate from another source. In this context, the high expression of a number of miRNAs in JKT-1, i.e. miR-17-5p, 20 (both part of one genomic cluster), 21, 106a, is interesting as these are reported as part of a miRNA signature for solid epithelial cancers (Volinia et al., 2006).

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article online:
Table S1.
Table S2.
Table S3.
This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2605.2007.00802.x
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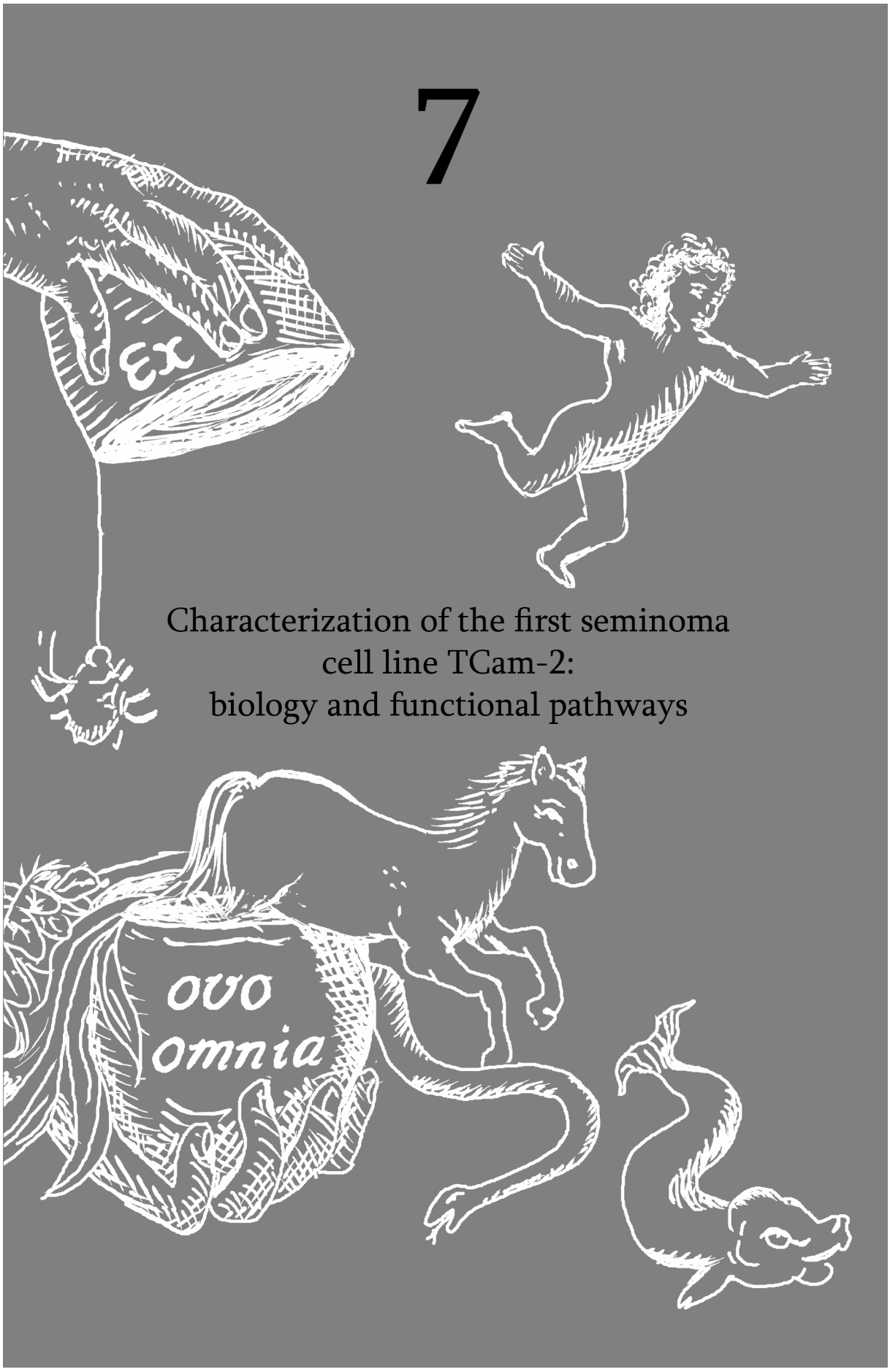

Characterization of the first seminoma cell line TCam-2: biology and functional pathways

Characterizion of the first seminoma cell line TCam-2: biology and functional pathways

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ABSTRACT

Testicular germ cell tumors of adolescents and adults (TGCTs) can be classified into seminomatous and nonseminomatous tumors. Various nonseminomatous cell lines, predominantly embryonal carcinoma (EC), have been established and proven to be valuable for patho-biological and clinical studies. So far, no cell lines have been derived from seminoma which constitute more than 50% of invasive TGCTs. Such a cell line is essential for experimental investigation of biological characteristics of the cell of origin of TGCTs, i.e. carcinoma *in situ* of the testis (CIS), which shows characteristics of a seminoma cell.

Before a cell line can be used as model, it must be verified regarding its origin and characteristics. Therefore, a multidisciplinary approach was undertaken on TCam-2 cells, supposedly the first seminoma cell line. Fluorescent *in situ* hybridization, array comparative genomic hybridization and spectral karyotyping demonstrated an aneuploid DNA content, with gain of 12p, characteristic for TGCTs. Genome wide (Affymetrix) mRNA and (Applied Biosystems) microRNA expression profiling supported the seminoma origin, in line with the biallelic expression of imprinted genes *IGF2/H19* and associated demethylation of the imprinting control region. Moreover, the presence of specific markers, demonstrated by immunohistochemistry, including (wild type) c-KIT, stem cell factor (SCF), placental alkaline phosphatase (PLAP), OCT3/4 and NANOG, and the absence of CD30, SSX2-4, and SOX2, confirms that TCam-2 is a seminoma cell line. Although mutations in oncogenes and tumor suppressor genes are rather rare in TGCTs, TCam-2 had a mutated *BRAF* gene (V600E), which likely explains the fact that these cells could be propagated *in vitro*.

In conclusion, TCam-2 is the first well characterized seminoma-derived cell line, with an exceptional mutation, rarely found in TGCTs.

submitted for publication

INTRODUCTION

Human testicular germ cell tumors of adolescents and adults (TGCTs) - the so called type II germ cell tumors (Oosterhuis and Looijenga 2005, for review) - originate from a transformed embryonic germ cell, either a primordial germ cell (PGC) or gonocyte. The precursor lesion is referred to as carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU) (Eble, et al. 2004; Skakkebaek 1972). The invasive tumors are subdivided clinically and histologically into seminomatous and nonseminomatous (NS) tumors. The NS can be subclassified into embryonal carcinoma (EC), teratoma (TE), yolk sac tumor (YST) and choriocarcinoma (CHC), representing both undifferentiated and differentiated embryonic and extra-embryonic lineages, respectively. Consistent with their cell of origin, the tumors show a unique combination of characteristics, like telomerase activity (Albanell, et al. 1999), expression of stem cell markers, including OCT3/4 and NANOG in seminoma and EC (Hoei-Hansen, et al. 2005; Looijenga, et al. 2003b), and in addition to these, SOX2 in EC (Korkola, et al. 2006), as well as an erased pattern of genomic imprinting (van Gurp, et al. 1994), and expression of the microRNA cluster hsa-miR 371-373, explaining the wildtype *TP53* in these tumors (Voorhoeve, et al. 2006). All invasive TGCTs are chromosomally aneuploid and exhibit specific patterns of losses and gains of genomic regions, with overrepresentation of 12p as the only consistent finding (Looijenga, et al. 2003c; van Echten, et al. 1995).

In 1975 the first type II TGCT-derived cell line was established by culturing EC cells (Fogh and Trempe 1975). Since then, multiple EC-derived cell lines have been generated, used in various studies (Andrews, et al. 2005). These have been very informative, although differences between various sub-lines, cultured in different laboratories, have been reported, for example regarding their pattern of X-inactivation (Looijenga, et al. 1997). In contrast to the relative wealth of independently generated EC-derived cell lines, only few reports on the establishment of cell lines representative for seminoma have been published (Kinugawa, et al. 1998; Mizuno, et al. 1993; von Keitz, et al. 1994). This is most likely due to the exceptional sensitivity of seminoma cells to undergo programmed cell death (apoptosis) upon disruption of their micro-environment (Berends, et al. 1991; Olie, et al. 1996), also known as anoikis (Frisch and Francis 1994). This might also explain why PGCs rapidly go into apoptosis when they stay extragonadal during normal embryonic development (Stallock, et al. 2003). So far, the seminomatous characteristics of the proposed seminoma cell lines have not been independently confirmed. In this context, we recently showed that the JKT-1 cell line, used in multiple studies as a model for seminoma, has none of the hallmarks of a classical seminoma (De Jong, et al. 2007), and is therefore not a seminoma cell line.

Already in 1993, the establishment of the cell line TCam-2 was reported (Mizuno, et al. 1993). This cell line originated from a primary testicular seminoma of a 35 year old patient and was generated by the traditional explant culture method, and succesfully transplanted into SCID mice. To proof it being representative for seminoma, we performed an extensive characterization of the TCam-2 cell line. The genomic constitution, imprinting status and immunohistochemical marker profile were determined, as well as genome wide (Affymetrix) mRNA and (Applied Biosystems) miRNA expression profiling. The results

show that TCam-2 cells contain all the typical features of seminoma. Furthermore, mutation analysis for several oncogenes and tumor-suppressor genes was done. In spite of a low mutation rate of these genes in TGCTs (McIntyre, et al. 2005; Sommerer, et al. 2005), the *BRAF* gene was mutated in TCam-2.

MATERIALS AND METHODS

Cell lines and primary tumor

TCam-2 cells were obtained from Sohei Kitazawa (Division of Molecular Pathology, Kobe University, Japan). The original formalin-fixed and paraffin-embedded tissues from the primary tumor could not be retrieved for investigation, and the patient data on clinical follow up were not available. The cells were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (Hyclone, Thermo Fisher Scientific, Etten-Leur, The Netherlands) and penicillin/streptomycin (Invitrogen, Breda, The Netherlands) at 37°C in a humidified cell culture incubator with 5% carbon dioxide. EC cell lines Tera-1 (Fogh 1978), NTera-2 (Andrews, et al. 1984), 2102Ep (Wang, et al. 1980) and NCCIT (Teshima, et al. 1988) were cultured as described before (Burger, et al. 1999). Cells were trypsined and harvested for the different experiments. Cytospins were prepared and DNA, RNA and protein were isolated (see below).

Fluorescent In Situ Hybridisation (FISH)

FISH was performed on metaphase spreads of TCam-2 cells as described before (Zafarana, et al. 2002). The following probes on chromosome 12 were used: PAC 876C13 (12p11.23), BAC RP11-268P4 (12p12.1) (the latter clone containing *EKI1*, a gene located at the telomeric region of the shortest region of overlap of amplification in seminomas: p11.2-p12.1 (Zafarana, et al. 2002)), and centromere specific probe Pα12H8 (Looijenga, et al. 1990). The centromere specific probe was labelled with biotin-16-dUTP (Roche, Mannheim, Germany) and detected using streptavidin-Cy3 (Jackson ImmunoResearch Laboratories, Cambridgeshire, UK), the other probes were labelled with digoxigenin-11-dUTP (Roche) and visualized with FITC-conjugated sheep anti-digoxigenin (Roche).

Spectral karyotyping (SKY)

Standard cytogenetic preparations were pretreated with RNase for 30 min at 37ºC and digested in 0.012% pepsin for 5 min. After washing, cells were fixed in 1% formaldehyde/50-mM $MgCl_2$ in PBS for 10 min, washed, and dehydrated. Hybridization and detection for SKY analysis was performed according to the manufacturer's protocol with minor adjustments (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Briefly, after two days of hybridization, slides were washed in 0.4 x SSC (pH 7.0) for 15 sec at 72°C followed by 2 x SSC at RT. Cells were counterstained with DAPI and mounted in antifade solution (Dabco-Vectashield 1:1). Using the SD300-C SpectraCube and Skyview analysis software (ASI) 10 metaphase cells were examined.

DNA isolation and Array Comparative Genomic Hybridization (array CGH)

Genomic DNA from the cultured cells was extracted as described before (Kersemaekers, et al. 2002). Briefly, each sample was treated with proteinase K followed by phenol/chloroform extraction and DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
TCam-2 cells and EC cell lines Tera-1, NTera-2, 2102Ep and NCCIT, were analysed on a human BAC/PAC array containing 3600 clones covering the full genome at on average a 1 Mb-spacing (Cardoso, et al. 2004). Labeling and hybridization were performed as described before (Cardoso, et al. 2004). Scanning was done on a ScanArray Express HT Microarray Scanner (Perkin-Elmer) and resulting images were analyzed with the GenePix Pro 5.0 software (Axon Instruments).

Mutation analysis of TP53, KRAS2, BRAF and c-KIT

To investigate the presence of TP53 mutations, in this case in exon 5 to 8, the Single-Strand Conformation Polymorphism approach as described before (Kersemaekers, et al. 2002) was applied. If aberrant bands after gel electrophoresis were identified, these were sequenced.
KRAS2, BRAF and *c-KIT* mutation analysis was done by Locked Nucleic Acid-mediated PCR Clamping and Melting Curve analysis followed by sequencing. For *KRAS2* primers, oligomer and probe were used as reported previously (Chen, et al. 2004). Oligonucleotides used for *BRAF* mutation analysis are the following: 5'-catctcagggccaaaaat in intron 14 (BRAF-F), 5'-tcctttacttactacacctcag in intron 15 (BRAF-R), 5'-gagatttc**a**ctgtagctagaccaa-Flu (sensor), 5'-LC Red 640-tcacctatttttactgtgaggtcttcatgaagaaa-Ph (anchor), gagatttc**t**ctgtagcta-Ph (LNA). For *c-KIT* the Lightcycler assay was adapted from Sotlar *et al.* (Sotlar, et al. 2003). The primers s (5'-cagccagaaatatcctccttact) and b (5'-ttgcaggactgtcaagcagag) were used in combination with the sensor probe 5'-agccagag**t**catcaagaatgattcta-Flu and the anchor probe 5'-LC Red640-atgtggttaaaggaaacgtgagtaccca-Ph. The LNA probe 5'-gccagag**a**catcaagaatg-Ph was used to suppress the WT allele (details of the method will be published elsewhere). In addition to the Lightcycler analysis, genomic DNA of TCam-2 was sequenced using forward (Intr16F 5'-gcaacactatagtattaaaaagttag) and reverse (18R1pna 5'-gactgctaaaatgtgtgatatccc) primers encompassing exon 17 of *c-KIT* (details of the method will be published elsewhere).

Expression of the imprinted genes IGF2/H19

To determine whether imprinted genes are either mono-allelically or bi-allelically expressed in the TCam-2 cells, *ApaI* and *RsaI* polymorphisms within the *IGF2* and *H19* genes were investigated. Therefore, DNA of cultured cells was amplified at both loci by PCR, followed by restriction enzyme digestion. In case heterozygosity was determined, a similar analysis was done on cDNA (van Gurp, et al. 1994). The following primers were used: 5'-cttggactttgagtcaaattgg-3' and 5'-gggtcgtgccaattacatttcat-3' (*IGF2* locus); 5'-tgactgaggaatcggctctggaag-3' and 5'-cggtcggagcttccagactag-3' (*H19* locus).

Methylation status of IGF2/H19 imprinting control region

The methylation status of the *IGF2/H19* ICR was determined by methylation-sensitive single nucleotide primer extension (MS-SNuPE)-PCR and sequencing of the CTCF core binding domain as described before (Sievers, et al. 2005). Primers for amplifying the CTCF core domain were 5'-tgtatagtatatgggtatttttggaggttt-3' and 3'-gggaataggatatttatagga-5'. The SNuPE primer sequence for the first cytosine in the CTCF binding domain was c_0-gttgtggaat(c/t)ggaagtggt (primer A) and for the first cytosine before the CTCF core domain was c_{12}-gaattggttgtagttgtggaat (primer B). Automated capillary electrophoresis and analysis were performed on an ABI PRISM 310 sequencer. MS-SnuPE profiles were confirmed by conventional bisulfite sequencing (Sievers, et al. 2005).

Expression analysis

Microarray expression profiling

Expression profile of the TCam-2 cell line was determined using Affymetrix GeneChip Human Genome U133 plus 2.0 array and analyzed using Omniviz and SAM software as described before (Looijenga, et al. 2006).

MicroRNA expression profiling

RNA highly enriched for low molecular weight (LMW) RNA species was isolated using the *mir*Vana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA quantity and quality was determined by a high-resolution electrophoresis bioanalyzer 2100 (Agilent, Amstelveen, NL) and by UV absorbance.

Expression of 300 mature miRNAs, including positive- and negative controls as well as normalization controls, was analyzed by TaqMan Multiplex MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. Briefly, single-stranded cDNA was synthesized in 7 pools of miRNA-specific RT-primers (each pool contains approximately 48 primers) from 20 ng of LMW RNA per pool in 20 µl reaction volume using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The reactions were incubated first at 16°C for 30 min and then at 42°C for 30 min. The reactions were inactivated by incubation at 85°C. The Multiplex RT products were diluted 10-fold by adding 180 µl H_2O. Each cDNA generated was amplified by Q-PCR using sequence-specific primers/probes on a fully automated Applied Biosystems 7900HT Real-Time PCR system. The 10 µl PCR included 5 µl Universal PCR Master Mix (No AmpErase UNG), 0.5 µl of sequence-specific primers/probes and 0.8 µl of diluted RT-product. The reactions were incubated in duplicate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Hsa-miR-16 /let-7, RNU-44 and RNU-48 were used as internal normalization controls. The median threshold value of the controls was determined and the difference with the target threshold was determined as delta Ct. This was used to investigate the different samples for expression of the targets along all samples included in the survey. Using the Omniviz tools, biostatistical analysis was performed on the total data set as described before (Looijenga, et al. 2006).

Immunohistochemistry

Immunohistochemical staining was performed on cytospin preparations and TCam-2 cells grown in 50 mm hydrophob LUMOX-dish (96077305, Greiner, Frickenhausen, Germany). General markers as vimentin (1:80; M725, DAKO, Glostrup, Denmark) and CAM5.2 (1:20; 345779, BD Biosciences, San Jose, CA, USA), and specific markers for various maturation stages of germ cell development like PLAP (1:200; 203, Cell Marque Corp, Hot Springs, AR, USA), OCT3/4 (1:1000; sc-5279, Santa Cruz Biotechnology, CA, USA), NANOG (1:800; AF1997, R&D Systems, Abingdon, UK), DAZL (1:750; Ab17224, Abcam, Cambridge, UK), VASA (1:1000; provided by D. Castrillon (Castrillon, et al. 2000)), TSPY (1:4000; provided by Y.-F. C. Lau (Lau, et al. 2000)) and SSX2-4 (1:100; provided by A. Geurts van Kessel (dos Santos, et al. 2000)) were applied. A standard 1-hour incubation at room temperature was used. Secondary biotinylated antibodies SWine-Anti-Rabbit (SWAR), Rabbit-Anti-Mouse (RAM) (both DAKO, Glostrup, Denmark) and Horse-Anti-Goat (HAG) (Vector Laboratories, Burlingame, CA, USA) were used in a 1:200 dilution and incubated for 30 minutes at RT. Antibody complex was visualised by avidin-biotin conjugated with horseradish peroxidase or alkaline phosphatase, respectively using 3,3-diaminobenzidine and New Fuchsin (72200, Fluka, Buchs SG, Switzerland) as chromogens, resulting in a brown and a red signal. All slides were counterstained with haematoxylin.

Protein isolation, Western Blotting and Immunoprecipitation

Isolation of protein and Western blotting were performed as described before (de Jong, et al. 2005). Immunoprecipitation of c-KIT (using rabbit polyclonal antibody, A4502 from DAKO) in TCam-2 protein lysate was done overnight at 4°C. Protein lysate from the F36P cell line was used as a positive control for c-KIT expression. Detection on the Western blot was done with the same antibody.

RESULTS

Genomic constitution of the TCam-2 cell line

To investigate the overall chromosomal constitution of the TCam-2 cell line array CGH and SKY analysis were performed. Array CGH of four well characterized EC cell lines was done for comparison. The hallmark of type II TGCTs, gain of the short arm of chromosome 12, was independently analyzed by FISH.

SKY analysis showed about 90 chromosomes with a complicated pattern of rearrangements, confirming the tetraploid status as described in the original paper (Mizuno, et al. 1993). Because of the large number of marker chromosomes no consensus karyotype was determined. A metaphase analysed by SKY is shown in Figure 1A to illustrate the genomic complexity.

The array CGH patterns of TCam-2 and for comparison the EC-cell lines Tera-2, NTera-2, 2102Ep and NCCIT were analysed. Several chromosomes of TCam-2 had array CGH

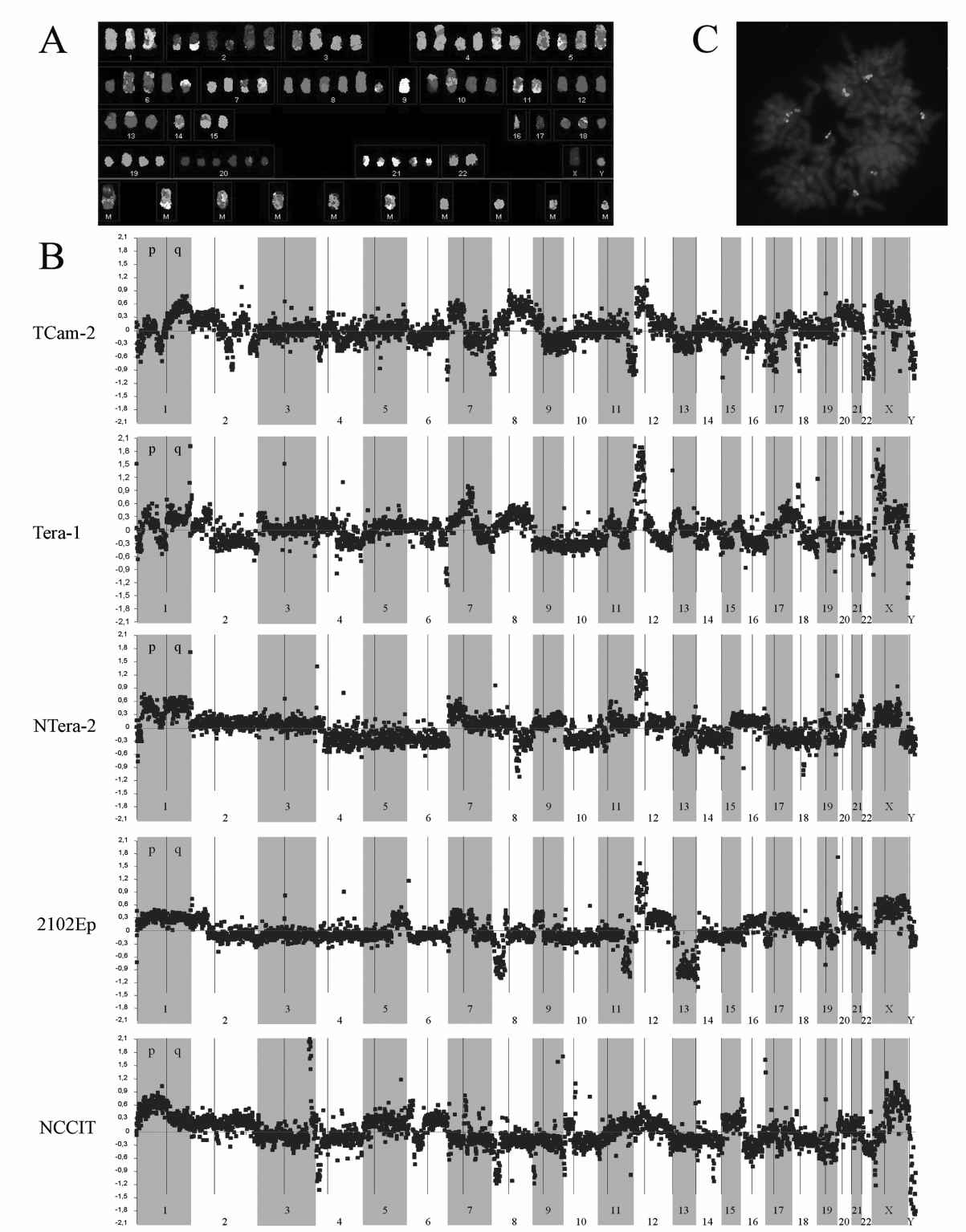

FIGURE 1. Genomic constitution of TCam-2 cells was determined by SKY analysis, array CGH and FISH. In addition, the EC-cell lines Tera-1, NTera-2, 2102Ep and NCCIT were analysed by array CGH. A) A representative metaphase of SKY analysis of TCam-2. The majority of chromosomes contains anomalies, and a large number of marker chromosomes is present; B) 3.6 K BAC/PAC array CGH analysis of TCam-2 cells and four EC-cell lines. Gain of 12p is present in all cell lines, while for example the Y chromosome is lost; C) FISH analysis confirmed the increased number of 12p copies (green signal) compared to the centromeric region of chromosome 12 (red signal) in TCam-2. *(Figure 1 colour image on page 184)*

profiles that were close to baseline (i.e. tetraploid): chromosome 3, 5, 6, 10, 14, 15, 16, 19 and 21. For other chromosomes, whole chromosomes or complete chromosome arms were gained (7p, 8q, 12p, 20) or deleted (9q, 13, 17p, 22 and Y). The following chromosomes contained gains or losses of a restricted region: 1, 2, 4, 7, 11, 18 and X. The characteristic chromosomal aberration of all invasive germ cell tumors, gain of 12p, was clearly present in TCam-2 cells as well as in all EC cell lines (Figure 1B). No other consistent patterns present in all cell lines, or when TCam-2 was left out, specific for EC cell lines, could be extracted. The specific gain of 12p in TCam-2 was confirmed by FISH analysis (Figure 1C).

Imprinting and methylation of the ICR

The pattern of genomic imprinting established during normal germ cell development (Surani 1998), has been used to characterize the embryonic germ cell origin of the seminomatous and the nonseminomatous TGCTs (van Gurp, et al. 1994). The polymorphisms within the *IGF2/H19* locus were used to study mono- or bi-allelic expression. In TCam-2 cells both *H19* and *IGF2* were biallelically expressed (Figure 2A) which implicates erasure of the genomic imprinting as is the situation in an immature germ cell (Szabo, et al. 2002; Szabo and Mann 1995). The imprinting status of the *IGF2/H19* locus is regulated by an imprinting control region (ICR) and the methylation status of the ICR is also informative for the status of genomic imprinting (Sievers, et al. 2005). In TCam-2 the ICR of the *IGF2/H19* locus was completely unmethylated (Figure 2B), as consistently found in seminomatous TGCTs (Sievers, et al. 2005).

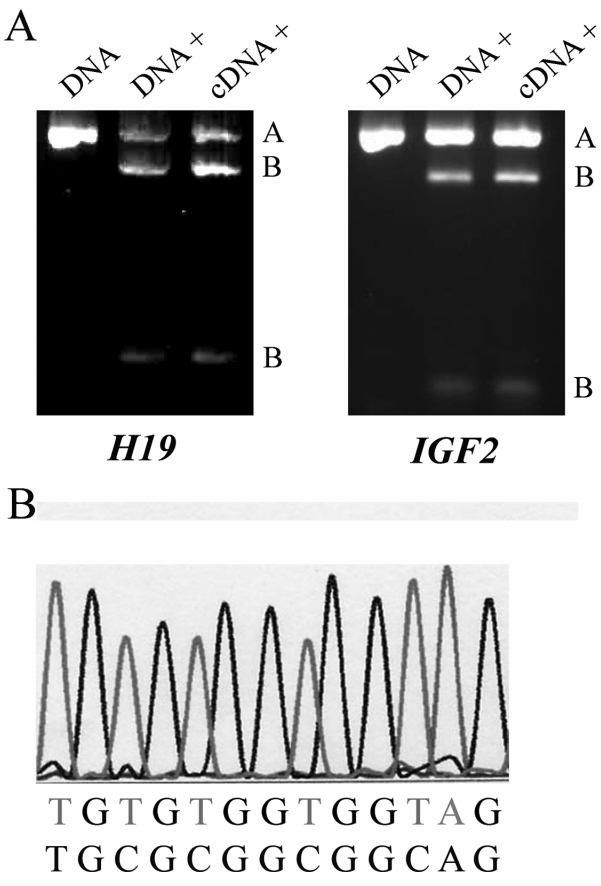

FIGURE 2.
The status of genomic imprinting in the *IGF2/H19* locus was determined by polymorphic restriction enzyme digestion and the methylation status of the imprinting control region (ICR) in this locus. A) In TCam-2 both *H19* and *IGF2* genes were informative as shown by *RsaI* and *ApaI* digestion on DNA derived PCR products (DNA+). The same restriction enzyme digestions of PCR products from TCam-2 mRNA showed biallelic expression of both *H19* and *IGF2* (cDNA+), indicated by both the A and B allele; B) Bisulfite sequencing reveals that the ICR in the *IGF2/H19* locus was completely unmethylated and all cytosines were converted to thymines (indicated in red compared to the original sequence as shown below). *(Figure 2 colour image on page 185)*

Marker expression profile

In addition to to gain of 12p and an erased pattern of genomic imprinting, seminoma and EC express a number of early embryonic markers. The one that has been demonstrated to be most informative in TGCT diagnostics, as well as for those tumors outside the testis, over the past few years is OCT3/4 (de Jong, et al. 2005). The presence of OCT3/4 in TCam-2 was demonstrated by immunohistochemistry, showing an intense nuclear staining of the cells grown in culture (Figure 3A left panel) and a specific 43 kD band in Western blotting analysis (data not shown). High expression of *OCT3/4* in TCam-2 comparable to the expression found in tumor samples of seminoma and EC is shown in Figure 3C (left panel), as determined by (Affymetrix) expression profiling. NANOG, another marker of pluripotency, with virtually the same expression pattern as OCT3/4 is highly expressed in TCam-2 as is the case in seminoma, CIS and EC (Hart, et al. 2005), determined by expression profiling and immunohistochemistry (data not shown). The embryonic stem cell marker SOX2, also involved in regulation of differentiation, was absent in TCam-2 cells and seminoma on protein and mRNA level, in contrast to the high expression found in EC (Figure 3B left upper panel and 3C middle panel). This is in line with its absence during normal germ cell development (unpublished observations). In addition, CD30 is a member of the tumor necrosis factor superfamily of cytokine receptors, which consistent expression in EC and EC-derived cell lines is related to inhibition of apoptosis (Herszfeld, et al. 2006). CD30 was also not expressed in TCam-2 cells on protein level (data not shown) nor on mRNA level demonstrated by (Affymetrix) profiling (Figure 3C right panel). Furthermore, TCam-2 cells showed expression of placental alkaline phosphatase (PLAP; Figure 3A middle panel), as well as direct alkaline phosphatase staining. Cytokeratin 8, as detected by CAM5.2 exhibited a dot-like staining pattern that can be observed in many seminoma cells (Figure 3A right panel). Finally, the stem cell factor receptor, c-KIT, and its ligand SCF were both expressed in the TCam-2 cells as determined by immunoprecipitation for c-KIT (Figure 3B left lower panel), Affymetrix expression profiling (data not shown), and immunohistochemistry (Figure 3B middle and right panel). Moreover, the markers specific for later stages of germ cell maturaton, i.e., SSX2-4 and DAZL were negative (data not shown). In contrast, VASA and TP53 showed a positive staining, the latter in a heterogeneous pattern. No expression was found for TSPY, due to the loss of the Y chromosome. Unsupervised hierarchical clustering of mRNA (Affymetrix) expression of a panel consisting of TCam-2 and TGCT tumor samples, showed that TCam-2 clustered in the seminoma-branch, separated from the EC samples (Figure 4).

In addition to the markers indicated above, a miRNA expression profile of TCam-2 cells was determined and compared to the EC cell lines. The different miRNAs included in the study were grouped into three classes based on their relative expression level compared to the normalized control: high expression (more than 2 SD), intermediate (-2 SD to 2 SD) and low (under -2 SD) (Table 1). The hsa-miRNA 371-373 cluster is specific for embryonic germ cells as well as for TGCTs, was highly expressed in TCam-2 indicating its TGCT origin (data not shown), and matching the presence of a wild type TP53 (Voorhoeve et al., 2006, see below). Comparison of the miRNA expression profile of TCam-2 to the various

EC cell lines showed a number of differentially expressed miRNAs. Especially, miRNA 145 and 324-5p are of interest here, because of their high expression relative to the EC cell lines and SOX2 as their predicted target.

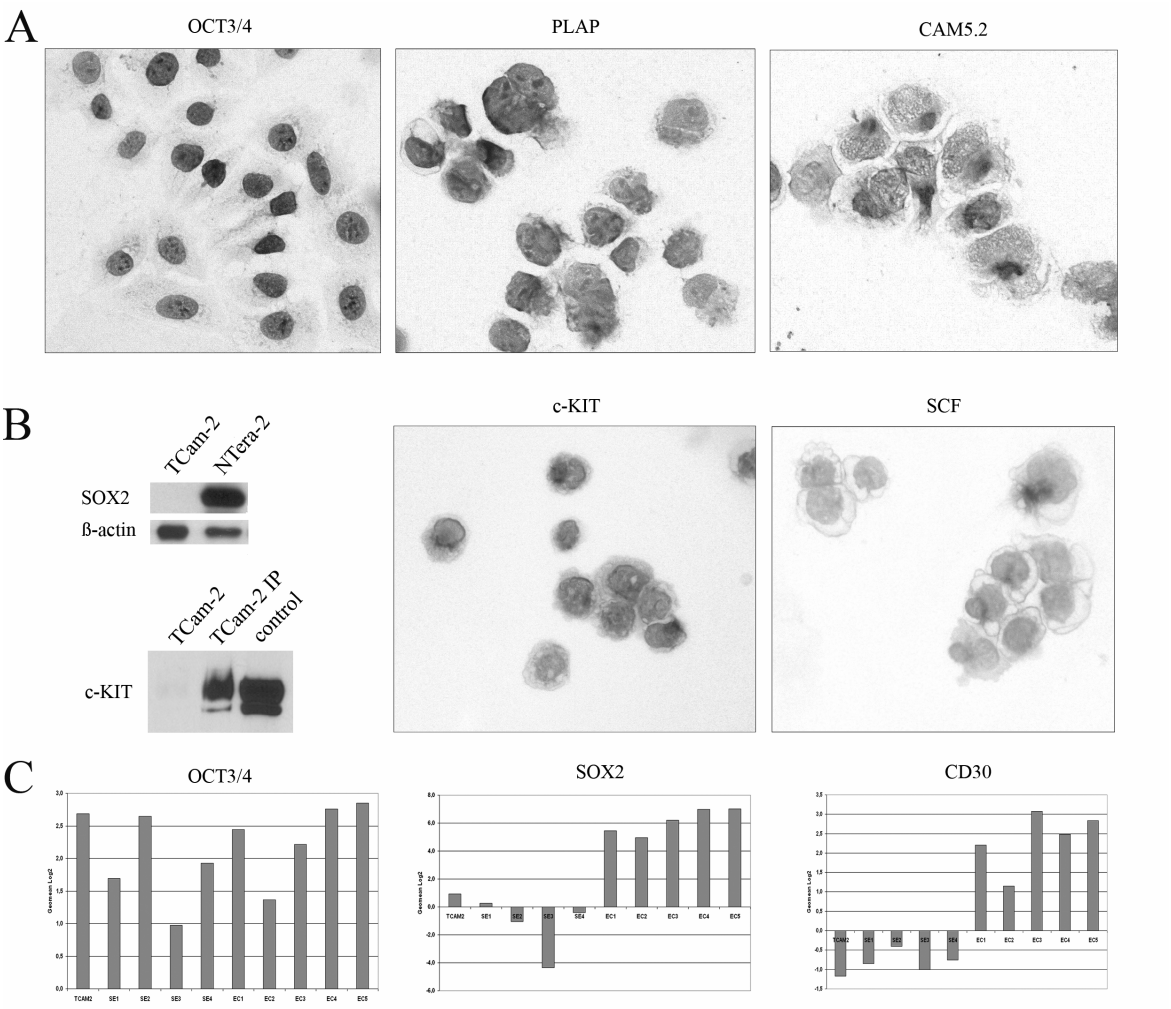

FIGURE 3. Expression of embryonic stem cell markers in TCam-2 and clinical germ cell tumor samples. A) left panel: Nuclear staining for pluripotency factor OCT3/4 in TCam-2 cells grown in a culture dish; middle panel: TCam-2 cells are positive for PLAP on cytospin preparation; right panel: dotted cytokeratin 8 (CAM5.2) staining in TCam-2 cells on cytospin preparation, as can be observed in seminoma; B) left upper panel: Western blot analysis shows expression of SOX2 in the embryonal carcinoma cell line NTera-2 and absence of SOX2 in TCam-2; left lower panel: Immunoprecipitation (IP) for c-KIT shows the expected band in TCam-2 and in F36P control total cell lysate, while c-KIT expression is very weak in total cell lysate of TCam-2; middle and right panel: immunohistochemistry for c-KIT and SCF on TCam-2 cells on cytospin preparations; C) Expression results of TCam-2 and primary human germ cell tumor samples based on genome wide (Affymetrix) expression profiling (log2 transformed data), left panel: OCT3/4 (expressed in all samples); middle panel: SOX2, and right panel: CD30, both expressed in embryonal carcinoma and low in seminoma and TCam-2. EC: embryonal carcinoma; DG: dysgerminoma; SE: seminoma. *(Figure 3 colour image on page 186)*

Mutation screening

Unlike other solid tumors, type II TGCTs have a low mutation rate in proto-oncogenes, including *BRAF* and *KRAS2* (Sommerer, et al. 2005), as well as the tumor suppressor gene

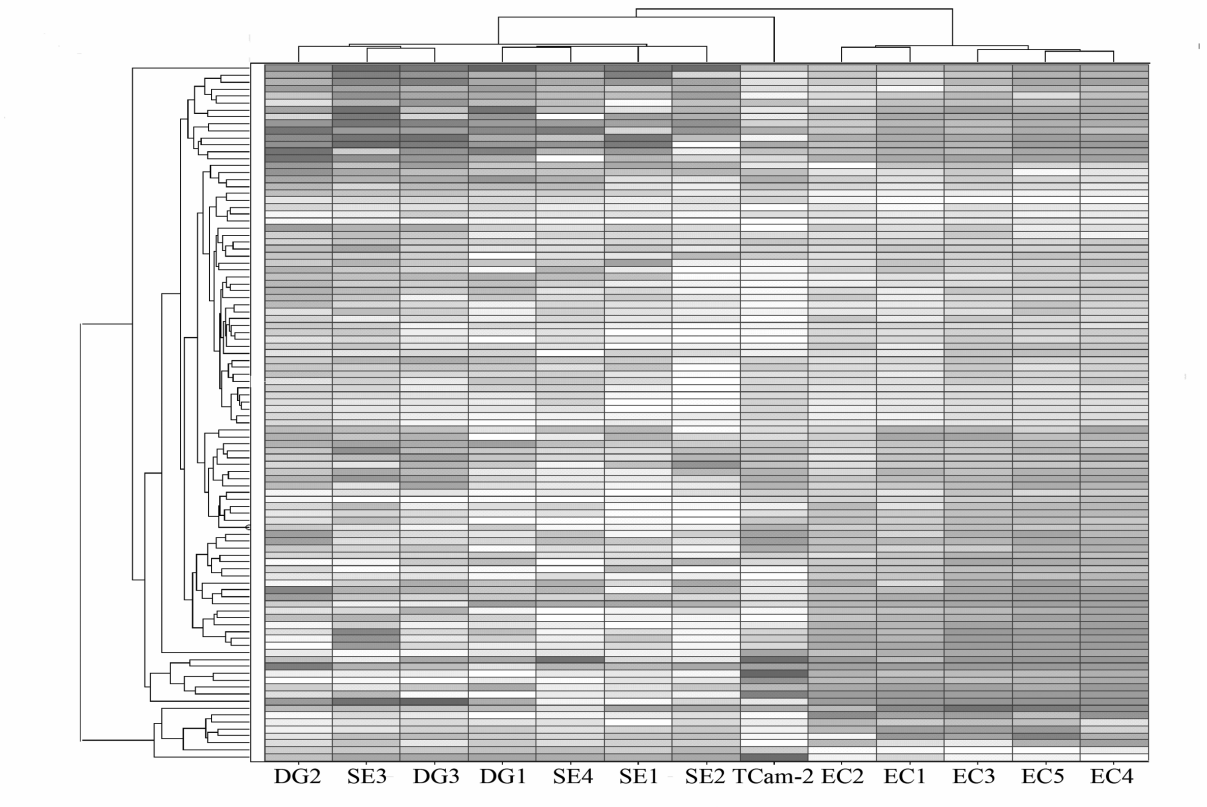

FIGURE 4. Unsupervised hierarchical clustering based on the top 100 differentiating genes between seminoma (SE)/dysgerminoma (DG) and embryonal carcinoma (EC) based on Affymetrix expression profiling to cluster the TCam-2 cell line (see supplementary data). TCam-2 clusters in the seminoma branch, separate from the EC samples. *(Figure 4 colour image on page 187)*

TABLE 1. MicroRNA expression profile of TCam-2

High

let-7e, let-7g, 9, 15b, 17-5p, 19a, 20a, 20b, 26a, 26aN, 28, 29a, 30a-5p, 30b, 30d, 31, 32, 92, 92N, 93, 99b, 124a, 126, 126#, 130b, 134, 135b, 142-5p, 145, 151, 154, 181a, 181b, 181d, 182, 186, 199a, 199a*, 199-s, 200bN, 200c, 205, 216, 217, 222, 301, 302a, 302d, 330, 342, 367, 371, 372, 373, 517a, 517c

Intermediate

7, 9*, 10a, 15a, 18a, 19b, 21, 23b, 23bN, 25, 26b, 27a, 27bN, 30a-3p, 30a-5pN, 34aN, 34b, 105, 106a, 108, 124b, 125b, 132, 135a, 139, 141, 142-3p, 152N, 183, 184, 187, 192, 196a, 197, 200b, 203, 218, 224, 220, 296, 302b, 302c#, 324-5p, 320, 321, 326, 328, 331, 337, 339, 340, 362, 365, 375, 380-3p, 381, 383, 423, 425, 452*,512-3p, 517b, 518a, 518b, 518c, 518f, 519c, 519d, 520g, 520h, 522, 525*

Low

let-7a, let-7c, let-7d, let-7i, 1, 10b, 16, 17-3p, 22, 23a, 24, 26bN, 27b, 29b, 29c, 30c, 30e-5p, 33, 34a, 34c, 34cN, 95, 96, 98, 99a, 100, 101, 103, 104, 106b, 107, 122a, 125a, 127, 129, 130a, 130aN, 133a, 133b, 137, 138, 140, 143, 144, 146a, 146b, 147, 148a, 148b, 149, 150, 152, 153, 154*, 155, 181c, 182*, 189, 190, 191, 193a, 193b, 194, 195, 196b, 198, 199b, 200a, 200a#, 200cN, 202, 202#, 204, 206, 208, 210, 210N, 211, 212, 213, 213, 214, 215, 219, 221, 223, 302a#, 302c, 323, 324-3p, 325, 325N, 299-3p, 299-5p, 335, 338, 345, 346, 361, 363, 368, 369-3p, 369-5p, 370, 373#, 374, 376a, 377, 378, 379, 380-5p, 382, 384, 409-5p, 412, 422a, 422b, 424, 429, 432, 432*, 433, 448, 449, 450, 451, 452, 453, 485-3p, 485-5p, 488, 489, 490, 491, 492, 493, 494, 496, 497, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512-5p, 514, 515-3p, 515-5p, 516-5p, 517*, 518a-2*, 518c*, 518d, 518e, 519a, 519e, 520a, 520a*, 520b, 520c, 520d, 520d*, 520e, 520f, 521, 523, 524, 524*, 525, 526a, 526b, 526b*, 526c, 527

A panel of 300 mature human miRNAs were divided in groups of high (more than 2SD), intermediate (-2SD to 2SD) and low (under -2SD) expression level. MiRNAs specific for GCTs are underlined.

TP53 (Kersemaekers, et al. 2002). In addition, TGCTs seldom have mutations in genes encoding receptor kinases (Bignell, et al. 2006; Greenman, et al. 2007). In contrast, mutations can be found in the stem cell factor receptor *c-KIT*, in particular in bilateral tumors (Looijenga, et al. 2003a). Mutation analysis of these aforementioned genes in TCam-2 cells showed a mutation in the proto-oncogene *BRAF* gene due a thymine to adenine transversion at nucleotide 1796 (T1796A), leading to a substitution of glutamic acid for valine at amino acid 600 (V600E)(data not shown). No mutations in *TP53, KRAS2* and *c-KIT* were found.

DISCUSSION

TGCTs are the result of erronous development of early germ cells during migration or upon their arrival in the gonad. In stead of progressing through the maturation process that will eventually form spermatozoa around puberty, these cells retain their embryonic phenotype and acquire malignant potential (Oosterhuis and Looijenga 2005). The transformation from the precursor lesion CIS to invasive TGCTs, seminoma and nonseminoma, is accompanied by gain of the short arm of chromosome 12, this feature being the genomic hallmark of all TGCTs irrespective of histology (Looijenga, et al. 2003c; van Echten, et al. 1995). The embryonic origin of TGCTs is reflected in their pluripotent potential: CIS and seminoma can progress to the undifferentiated EC – a process called reprogramming - , and these cells can recapitulate normal development by differentiation to embryonic (teratoma) and extra-embryonic tissues (yolk sac tumor and choriocarcinoma), as well as the germ lineage (Honecker, et al. 2006). Besides their pluripotency, TGCTs harbour a defined set of features of embryonic development, some of which have been used for diagnostic purposes. The most striking example is the identification and current use as diagnostic marker of OCT3/4 (de Jong, et al. 2005).
In this study we characterized the cell line TCam-2, supposedly derived from a seminoma, by determining whether it harbours the genomic and embryonic hallmarks of invasive TGCTs. Our results showed specific gain of 12p in TCam-2 cells by SKY, array CGH and FISH. Expression of the (embryonic) miRNA cluster hsa-miR 371-373, as well as protein and mRNA expression of embryonic stem cell markers OCT3/4 and NANOG, together with biallelic expression of imprinted genes *IGF2* and *H19* proved the TGCT origin of TCam-2. The seminomatous origin of TCam-2 was reflected by the expression of c-KIT, SCF and PLAP, and by the absence of SOX2 and CD30. In addition, the tetraploidy of TCam-2 cells was close to the hypertriploid number of chromosomes found in primary seminomas, in contrast to the hypotriploidy of nonseminomas (Oosterhuis, et al. 1989). Our results are consistent with the recent array CGH data and the reported OCT3/4 protein expression in TCam-2 (Goddard, et al. 2007).
The proven seminomatous origin of TCam-2 paves the way to research that could not be undertaken previously because of lack of a valid model for seminomas. Seminomas are considered the default developmental pathway of CIS cells, or stated otherwise: a proliferation of CIS cells that have acquired invasiveness, possibly related to gain of 12p. Therefore, the availability of a seminoma cell line offers opportunities to study earlier

steps in germ cell tumorigenesis than in the EC derived cell lines. Especially the transformation step of CIS/seminoma cells to EC cells, which results in the clinically unfavorable nonseminomas, could not be explored until now. This process called reprogramming is a black-box during which the CIS/seminoma cells acquire the expression of stem cell markers SOX2, CRIPTO-1/TDGF1 and CD24 (Korkola, et al. 2006; Looijenga, et al. 2007), resulting in the undifferentiated EC cells. Artificial upregulation of these genes in TCam-2, combined with downregulation of CIS/seminoma specific genes by siRNA, can be used to mimic the process of reprogramming and break it down into a molecular biological sequence. Interestingly, the miRNAs 145 and 324-5p might be gatekeepers in this process, by preventing translation of SOX2 mRNA in TCam-2. In addition, the proposed role of TSPY in the development of these tumors can be investigated (Kersemaekers, et al. 2005; Li, et al. 2007a; Li, et al. 2007b)

The favorable treatment outcome in most TGCTs, even after metastasizing, is tightly linked to the exceptional sensitivity to chemotherapy and radiation (Kollmannsberger, et al. 2006). The absence of G1 arrest in response to DNA damage, is another feature that reflects their embryonic origin (Aladjem, et al. 1998). The sensitivity of TCam-2 to different chemotherapeutics and different radiation dosis regiments can now be tested *in vitro*. Besides *in vitro*, TCam-2 can also be studied *in vivo* as xenografts in SCID mice, as was demonstrated in the original article (Mizuno, et al. 1993). In this way, behavior of the cells injected in the testis and other locations can be used to study, for instance, metastasizing capacity.

Although the establishment of TCam-2 creates a new research area to be explored, the fact that it is the only proven seminoma cell line, is reason for caution. If TCam-2 was derived from a typical seminoma, then it is expected that it could not have been established by a traditional culture method, as has been done. Therefore, the initial tumor cells probably had an advantage that helped them escape from the anoikis that normally awaits seminoma cells upon disruption of their microenvironment (Olie, et al. 1996). A possible explanation for the succesfull establishment of this unique cell line was found in the mutation analysis of TCam-2. Although primary GCTs generally have a low mutation frequency in well-known tumor suppressor and oncogenes as *TP53, KRAS2* and *BRAF*, GCT-derived cell lines show a relatively high proportion of mutations. For example, *TP53* is mutated in NCCIT (Burger, et al. 1998), GCT85 has mutated *BRAF*, and 833KE has mutant *KRAS2* (unpublished observations). In TCam-2, *BRAF* was mutated, leading to a constitutively activated RAS-pathway (McIntyre, et al. 2005). This mutation could be the explanation that these cells could be propagated *in vitro*. Because of the lack of the primary tumor, it could not be determined whether this mutation was present in the original tumor, or generated due to *in vitro* culturing. Our hypothesis that *in vitro* growth of TCam-2 cells is possible due to the *BRAF* mutation finds support in our own experience of trying to establish seminoma cell lines. Typically seminoma cells go rapidly into apoptosis upon disruption of their microenvironment (Olie, et al. 1996). However, the presence of either a *RAS* mutation or high level amplification of 12p sequences results in prolonged *in vitro* survival (Roelofs, et al. 2000). Both events make the tumor cells more resistent to apoptosis, supposedly in a similar way as the *BRAF* mutation in TCam-2.

In conclusion, this study demonstrates that the TCam-2 cell line has all the essential features to qualify as a valid model for seminomatous tumors. The presence of an activating mutation in the *BRAF* oncogene provides a possible explanation why this cell line could be established.

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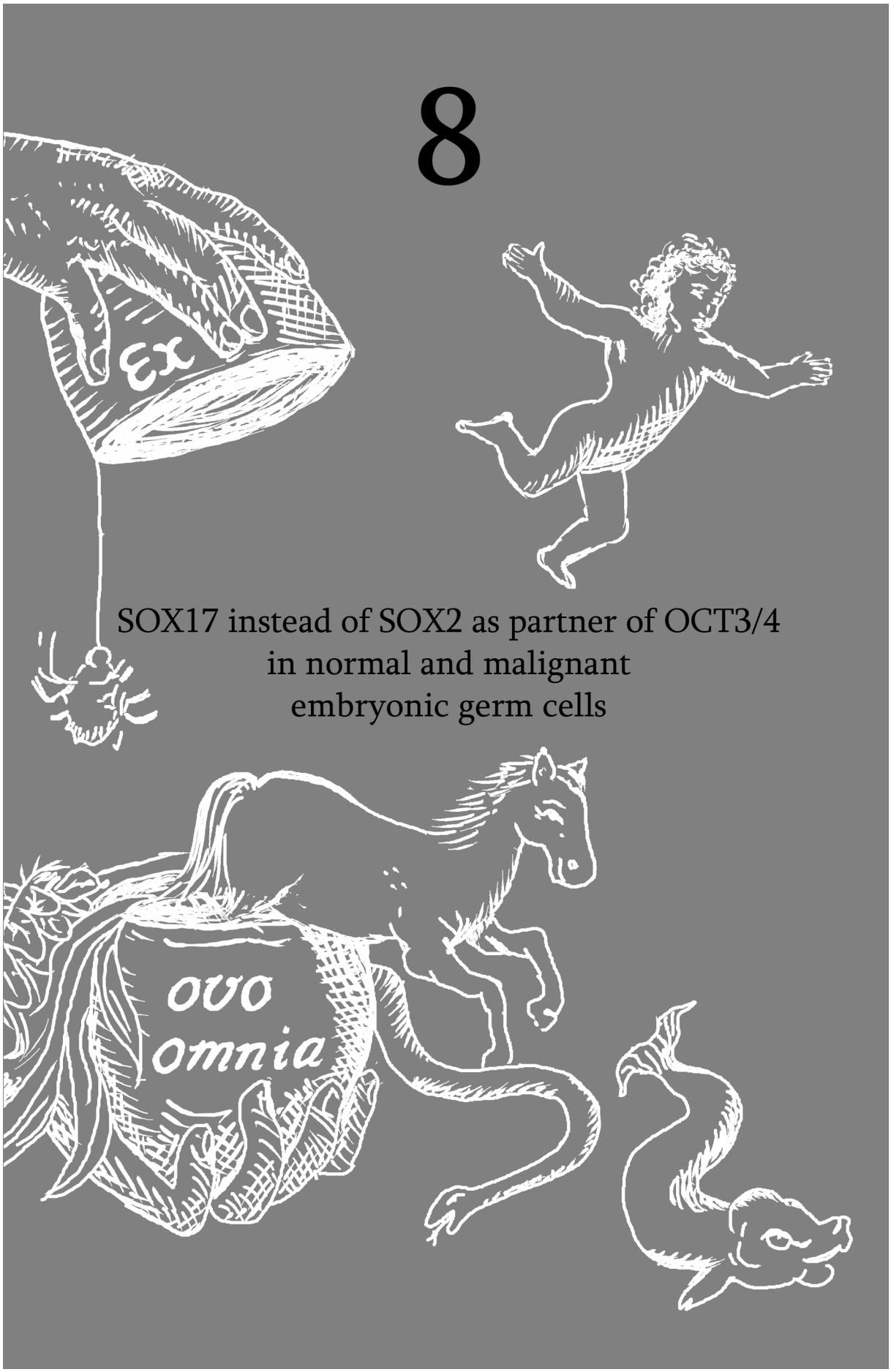

SOX17 instead of SOX2 as partner of OCT3/4
in normal and malignant
embryonic germ cells

SOX17 instead of SOX2 as partner of POU5F1 (OCT3/4) in normal and malignant embryonic germ cells

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ABSTRACT

Embryonic development is characterized by a spacial and temporal sequence of tightly regulated differentation, starting from a population of pluripotent stem cells. The combined action of SOX and POU families of transcription factors plays major roles in this regulation by forming heterodimers consisting of a SOX protein and a POU protein bound to the target DNA sequence. In embryonic stem cells the protein interaction between SOX2 and POU5F1 (also known as OCT3/4 and OCT4) is essential for maintaining the undifferentiated state by activating pluripotency-linked genes, and inhibition of genes involved in the various differentiation pathways. Besides embryonic stem cells, POU5F1 is also present during normal development in migrating primordial germ cells and the undifferentiated germ cells in the gonad, the gonocytes. However, the target genes of POU5F1 in germ cells and how these genes are regulated remains to be studied. Here we show that SOX2 is absent in germ cells of human fetal gonads, carcinoma *in situ* (CIS), i.e. the precursor lesion of testicular germ cell tumors of adolescents and adults (TGCTs), and seminoma, the latter confirming other studies. Based on genome wide expression profiling, SOX17 was found to be the alternative protein partner for POU5F1 in early germ cells and their malignant counterparts, CIS and seminoma. Immunohistochemistry, Western blot analysis and quantitative RT-PCR showed that SOX17 is a suitable marker to distinguish seminoma from embryonal carcinoma, the latter being the malignant counterpart of embryonic stem cells, and SOX2 positive. This is confirmed in representative cell lines. Furthermore, we show that aberrant SOX2 expression in Sertoli cells is strongly associated with the presence of CIS in the seminiferous tubules.
In conclusion, this study demonstrates the absence of SOX2 in human embryonic and malignant germ cells, which express SOX17 in conjunction with POU5F1. This finding has both diagnostic as well as developmental biological implications.

submitted for publication

INTRODUCTION

The SOX family of transcription factors is involved in orchestrating development from the early cells in the embryo to differentiated lineages of specialized cells. Currently, twenty SOX proteins have been identified in humans, and all have a high-mobility group domain (HMG), a 79-amino acid protein motif, that binds into the minor groove of the DNA helix and will induce DNA bending, involved in expression regulation [1]. SOX proteins have at least 50% amino acid similarity to the HMG domain of SRY, the testis-determining factor in mammals excluding monotremes [2], and are therefore named SRY-related HMG box (SOX) proteins. Although different SOX proteins can bind highly similar DNA sequences *in vitro*, they are expressed in a cell-type specific manner in which they regulate their target genes [3]. This regulation is achieved by the collaboration of SOX proteins in association with other proteins [4]. Abundantly represented among the protein partners are the POU homeodomain proteins [5, 6]. Like SOX proteins, these transcription factors are expressed in a cell-specific way and interact simultaneously with the DNA and stabilizing partner proteins [7, 8]. The joined action of SOX and POU proteins has been confirmed in various cell-types, for example the activation of *PAX6* by SOX2 and POU2F1 in lens placode formation [9], and the combined action of SOX10, POU3F2 (also known as BRN-2 and N-OCT3) and POU3F1 (OCT6) in expression control of *KROX20* in the myelination of Schwann cells [10]. In oligodendrocytes the interaction between SOX11 and POU3F3 (BRN-1) has been proven, however the first target gene of this couple remains to be identified [11].

The most extensively studied SOX-POU partnership is that of SOX2 and POU5F1 (also known as OCT3/4 and OCT4) in the inner cell mass of early embryonic development and the *in vitro* counterpart, i.e., embryonic stem (ES) cells. These proteins have been shown to dimerize in a target-specific conformation onto the DNA [12], and regulate their own gene expression as well as that of their protein partner [13-15]. Both factors are in the center of an intricate network required to maintain pluripotency [16-18 for review], where feedback mechanisms control the appropriate level of the transcription factors [19], and deviation will promote differentiation [20, 21]. Genome wide screens in human and mouse ES cells have identified a large number of target genes that are activated or repressed by simultaneous binding of POU5F1 and SOX2 depending on their involvement in pluripotency or differentiation [22, 23]. However, a substantial number of target genes in ES cells are only bound by either POU5F1 or SOX2, and no cooperative action in the control of these target genes is expected.

Besides ES cells, POU5F1 is present in primordial germ cells (PGCs) which arise from the proximal epiblast cells of the bi-layered embryo [24, 25]. During migration of PGCs, *POU5F1* remains highly expressed [26] and after arrival at the gonadal ridge (the PGCs are now called gonocytes) these cells progressively lose POU5F1 upon maturation during gametogenesis. Interestingly, knock-out of *POU5F1* in mouse PGCs results in induction of apoptosis, instead of differentiation as in ES cells [27]. Normally, one year after birth no POU5F1 positive cells are present either in the gonads or any other organs and tissues. The mechanistic basis of the different function between POU5F1 in PGC and ES cells remains to be elucidated.

In humans prolonged expression of *POU5F1* is strongly associated with development of malignant testicular germ cell tumors (TGCTs), clinically manifest after puberty. These so called type II TGCT, i.e., seminomas and nonseminomas, arise from a malignantly transformed PGC/gonocyte and this dormant precursor lesion is named carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (IGCNU) [28, for review]. The precursor lesion of dysgenetic gonads is known as gonadoblastoma [29, 30]. After puberty, proliferation of these precursor cells will give rise to invasive seminoma (known as dysgerminoma in the ovary and dysgenetic gonad), the default pathway for CIS cells, or – by acquiring additional expression of ES cell markers - to invasive embryonal carcinoma (EC), the undifferentiated cells of nonseminoma, representing the inner cell mass during normal embryogenesis. Furthermore, EC cells can differentiate into teratoma (the somatic lineage) and the different extra-embryonal lineages, i.e., yolk sac tumor and choriocarcinoma, as well as the germ cell lineage. During the past years, POU5F1 has proven to be a specific marker in TGCT diagnostics with 100% of CIS, seminoma and EC cells, showing a profound nuclear staining [31-33].

The physiological function that POU5F1 exerts in different cell types, is dependent on the cell-specific target genes and therefore, on the selected POU5F1 protein partner in that specific cell type. The role of SOX2 interaction with POU5F1 in maintaining pluripotency and preventing differentiation is well established [13], however, how POU5F1 expression is involved in apoptosis control in PGC is not clear. To gain more insight into this process, we studied the expression of SOX2 in germ cells in early embryonic gonads to adult testis and ovary, and in pre-invasive and invasive TGCTs by immunohistochemistry. This shows that SOX2 is not present during normal human germ cell development and the malignant counterpart, CIS and seminoma cells, but indeed present in EC, as well as heterogeneously in different derivatives. Furthermore, we show that SOX2 can be expressed in Sertoli cells associated with the presence of CIS cells in seminiferous tubules, which is a significant observation, because it may result in overdiagnosis of intratubular EC. Based on genome wide mRNA expression profiling and quantitative RT-PCR of patient-derived TGCT samples, we identified another SOX family member, SOX17, as the most obvious candidate to interact with POU5F1 in CIS and seminoma cells. SOX17 plays a role in endoderm formation, oligodendrocyte differentiation and has been reported to function as a transcriptional activator in mouse pre-meiotic germ cells [34-36]. Moreover, most recently, it has been found to distinguish embryonic from adult hematopoietic stem cells [37]. Immunohistochemistry of SOX17 demonstrated nuclear co-expression with POU5F1 in fetal gonocytes, CIS and seminoma cells, as well as in the recently characterized seminoma cell line TCam-2, but not in EC and derived-cell lines. This finding allows distinction between seminoma and EC in a diagnostic setting, for which so far no absolute marker for seminoma is available. In addition, this knowledge will deepen our understanding of the progression of TGCTs from CIS to either seminoma or EC.

MATERIALS AND METHODS

Immunohistochemistry

The presence of SOX2 in normal and pathological gonadal development was studied by immunohistochemistry using a monoclonal antibody (AF2018, R&D Systems, Abingdon, UK) in a 1:750 dilution incubated for 2 hours at RT. As a secondary antibody, a biotinylated rabbit anti-mouse antibody was applied (1:200; E0413, Dako, Glostrup, Denmark) and bound antibody complex was visualized using the horseradish peroxidase avidin-biotin complex (HRP-ABC) method. The samples examined are listed in Table 1. In addition, double staining was performed using a monoclonal antibody directed against POU5F1 (1:1000; sc-Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SOX2, respectively, with 3-amino-9-ethyl-carbazole (A5754: Sigma, Steinheim, Germany)/H_2O_2 for a red staining and Fast Blue/Naphtol AS-MX phosphate (F3378 and N500: Sigma) for a blue staining. Detection of SOX17 in gonadal development, normal spermatogenesis and TGCTs was done with a goat polyclonal antibody (GT15094, Neuromics) in a 1:3000 dilution for 2 hours at RT. Subsequently, a biotinylated horse anti-goat secondary antibody (1:200; BA-9500, Vector) was used, and detection was done using the HRP-ABC method. Antibodies against SOX17 from Santa Cruz (sc-17355) and R&D Systems (MAB1924) were also tested, but these gave non-specific and cytoplasmic staining. Therefore, these antibodies were not used for further experiments.

TABLE 1. Gonadal samples used for SOX2 immunohistochemistry

Fetal testis: N=16 (age in weeks) [60]: 15, 16, 16, 16, 17, 17, 18, 18, 21, 21, 23, 24, 27, 30, 38, 40

Fetal ovary: N=5 (age in weeks)[61]: 18, 20, 26, 33, 41

Adult testis: N=1: 42 years (additional samples are included in the TGCT TMA (see below))

Post-natal ovary: N=5 (age in years): 11, 31, 33, 36, 50

Type II TGCTs: (tissue micro array; TMA):
CIS (N=39), seminoma (N=50), EC (N=42), yolk sac tumor (N=8), choriocarcinoma (N=13), teratoma (N=26)

Dysgenetic gonads and GCTs N=9 [62]:

Published code	*age (years)*	*Tissue*	*Tumor*
32r	16	undifferentiated gonadal tissue (UGT)	gonadoblastoma (GB)
5l**	14	UGT	GB+dysgerminoma (DG)
31l	14	Streak	-
31r	14	Streak	-
39r	19	UGT	GB+DG
39l	19	UGT	GB
3l	1 month	testis+ovary	-
3r	1 month	ovary+UGT	-
29r	14	Streak	GB

In addition to fetal gonads indicated in Table 1, a series of Bouin fixed early fetal gonads (57 to 63 days) was obtained from Phillipa Saunders and Richard Anderson (Centre for Reproductive Biology, Queen's Medical Research Institute, The University of Edinburgh,

UK). Extensive testing using an appropriate positive control showed that the SOX2 antibody (AF2018, R&D Systems) did not work on Bouin fixed material, and therefore only SOX17 (GT15094, Neuromics) immunohistochemistry could be performed on these samples.

Cell culture

The cell line TCam-2, recently confirmed to be a seminoma cell line (De Jong, submitted for publication), was obtained from Sohei Kitazawa (Division of Molecular Pathology, Kobe University, Japan). The cells were cultured in RPMI 1640 (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (Hyclone, Thermo Fisher Scientific, Etten-Leur, The Netherlands) and penicillin/streptomycin (Invitrogen, Breda, The Netherlands) at 37°C in a humidified cell culture incubator with 5% carbon dioxide. The EC cell lines Tera-1 [38], NTera-2 [39], 2102Ep [40], 833KE [41] and NCCIT [42] were cultured as described before [43]. The cell line JKT-1, recently demonstrated to be not a seminoma cell line was cultured as described before [44]. These cells likely represent a later stage of germ cell development. Cells were trypsinized and harvested for the different experiments. Cytospins were prepared and RNA was isolated (see below).

Protein isolation and Western blot analysis

Isolation of protein and Western blot analysis were performed as described previously[32].

Quantitative RT-PCR for SOX2 and SOX17 in normal testis and TGCTs

High-quality total RNA was extracted from normal testis (n=4) and patient derived TGCT samples containing either CIS (n=4), seminoma (n=12) or EC (n=11) using RNAqueous-4PCR kit (Ambion Europe, Huntingdon, United Kingdom) according to the manufacturer's instructions. In addition, RNA from cell lines JKT-1, TCam-2 and the EC cell lines Tera-1, NTera-2, 2102Ep, and NCCIT was used. Samples were treated with DNase and checked for residual DNA contamination by PCR. Quantitative PCR was done using the real-time PCR ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA). For detection of SOX2 expression the following primers-set was used: SOX2-F 5'-CACACTGCCCCTCTCACACAT-3' and SOX2-R 5'-CATTTCCCTCGTTT-TTCTTTGAA-3'. Quantitative values were obtained from the C_t. SOX2 mRNA was quantified relative to HPRT (SOX2 mRNA value = $2^{(\text{mean Ct HPRT} - \text{mean Ct SOX2})}$) [45]. For detection of SOX17 the following primers were used: SOX17-F (exon 1) 5'-GATGCGGGATACGCCAGTGAC-3' and SOX17-R (exon 2) 5'-GCTCTGCCTCC-TCCACGAAG-3' and quantified by the formula described above.

Micro-array expression profiling

Expression profile of frozen TGCTs (five spermatocytic seminomas, three dysgerminomas, being the ovarian counterpart of seminoma [45] four seminomas, and five ECs), cell line JKT-1 and the seminomatous TCam-2 cell line was determined using Affymetrix GeneChip Human Genome U133 plus 2.0 array as described before [45]. Analysis was done

using Omniviz and SAM software as reported in the same manuscript. Expression levels of each SOX family member were analyzed for all samples and plotted in a graph.

RESULTS

SOX2 is not expressed in normal human gonadogenesis

The gate-keeper of pluripotency in ES cells, POU5F1, switches its function to a survival factor in early migrating germ cells, and loss of POU5F1 in these cells induces apoptosis [46]. In ES cells and their malignant counterpart, EC cells, SOX2 is regarded as the well-established protein partner of POU5F1. To elucidate whether SOX2 plays a role in germ cell development and gonadogenesis, immunohistochemistry was done on gonads from early fetal life (15 weeks) to adult ovary and testis. The SOX2 protein was not expressed in germ cells regardless of their state of maturation (data not shown). Occasionally, SOX2 positive Sertoli cells were present in normal adult testis, however, the expression level of SOX2 mRNA was very low (Figure 1A).

SOX2 in testicular germ cell tumors

The differential expression of SOX2 between seminoma and EC cells is used as an argument to explain the differences in pluripotency between these cells [47, 48]. The relative overexpression of several core "stemness" genes, like *EBAF*, *TDGF1*, and *SOX2*, reflects the undifferentiated state of EC cells and is the starting point of their differentiation potential, analogous to the ES cells derived from the inner cell mass of early embryonic development.
To study SOX2 expression in the development of TGCTs, tissue micro-arrays (TMA) of the various invasive histological subtypes and the precursor lesion CIS were used (see Table 1). As expected, all EC cells were positive for SOX2, while CIS, seminoma, yolk sac tumor and choriocarcinoma were consistently negative. In teratoma a small number of SOX2 positive cuboidal or columnar epithelium cells of endodermal origin were present as was recently also reported in teratomas of the central nervous system [49]. However, in the seminiferous tubules containing CIS cells (always POU5F1 positive) a large number of SOX2 positive cells were seen more luminal situated than the CIS cells (Figure 1B). Detailed morphological study of these cells and double staining for SOX2 and vimentin showed that they are Sertoli cells (Figure 1C). Quantitative RT-PCR confirmed the elevated expression of SOX2 in samples containing CIS cells, compared to normal testis (Figure 1A).

SOX17 is expressed in CIS, gonadoblastoma and seminomatous cells and not EC

With the absence of SOX2 expression in germ cells and their malignant counterparts, CIS and seminoma cells, the question arises whether POU5F1 is interacting with another SOX member, and thereby regulating its target genes in these cells. To gain insight in this possibility, we screened the expression profiles of all SOX family members in TGCT

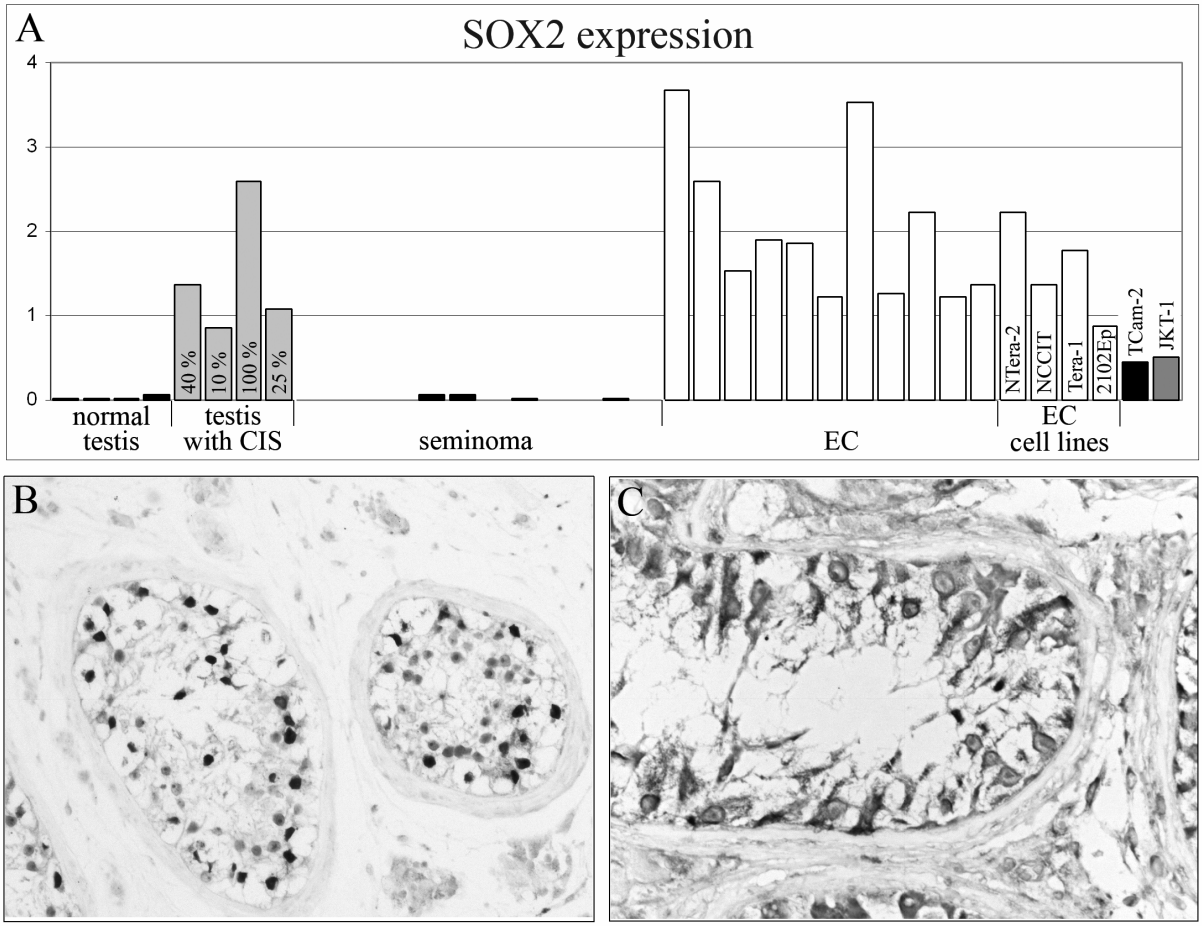

FIGURE 1. A) Quantitative RT-PCR for SOX2 on TGCT samples. Tested are the cell line JKT-1, the seminomatous cell line TCam-2 and the EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep). A high expression of SOX2 is observed in the EC samples and cell lines. In testis parenchyma containing CIS (percentage is indicated in the bars) the SOX2 mRNA expression is elevated compared to normal testis; the level of expression parallels the amount of CIS containing seminiferous tubules. TCam-2 and JKT-1 show a level of expression in between normal testis/seminoma and EC samples and cell lines. B) Double immunohistochemistry for SOX2 (red) and POU5F1 (blue) shows that SOX2 is not expressed in the POU5F1 positive CIS cells, but in a cell type more luminally located, being Sertoli cells. C) Double staining for Vimentin (blue) and SOX2 (red) combined with morphology and the position in the seminiferous tubule shows that the SOX2 positive cells are Sertoli cells. *(Figure 1 colour image on page 188)*

samples and the cell lines JKT-1 and TCam-2 (Figure 2). The expression pattern of SOX2 was confirmed on the mRNA level, being specifically expressed in EC. Most of the *SOX* members gave a non-specific pattern for the different histological elements investigated, including the cell lines, with one interesting exception. *SOX17* is highly expressed in all seminoma/dysgerminoma samples, as well as the seminoma cell line TCam-2, and virtually absent in EC cells (absolute value for specific probe sets below 30). The mRNA expression profile of SOX17 in POU5F1 positive TGCTs (seminoma/dysgerminoma and EC) is exactly complementary to that of SOX2. Therefore, we subsequently focused on this specific SOX member for the rest of the study. Quantitative RT-PCR confirmed the elevated SOX17 expression in seminoma samples and seminoma cell line TCam-2 compared to EC samples and EC cell lines (Figure 3A). Immunohistochemistry using a specific antibody showed the nuclear expression of SOX17 in all CIS/gonadoblastoma and

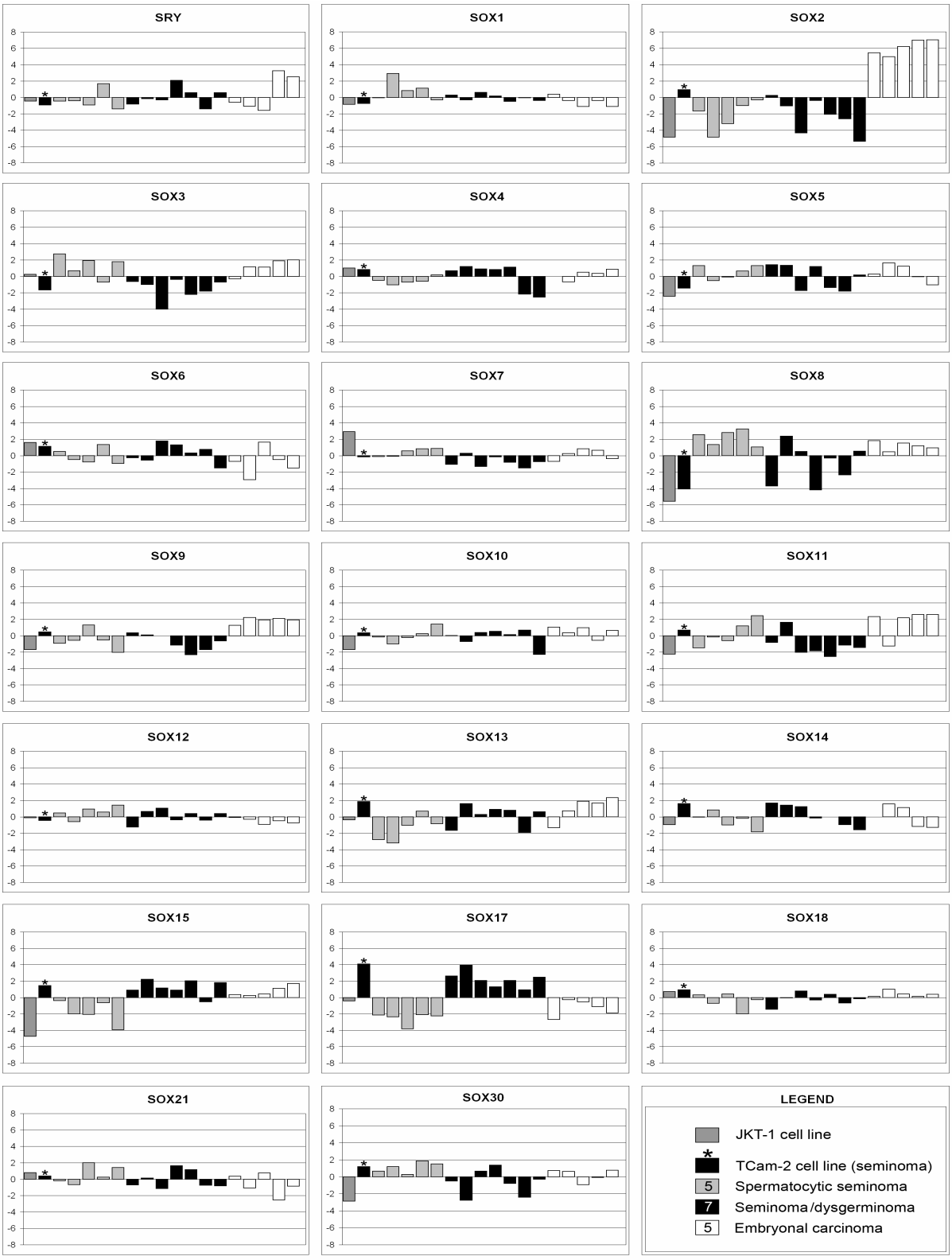

FIGURE 2. Expression results of all 20 human SOX family members in TGCT samples and cell lines JKT-1 and TCam-2 based on genome wide (Affymetrix) expression profiling (log2 transformed data). The following samples were used: five spermatocytic seminomas, four seminomas, three dysgerminomas and five EC (also indicated in the legend in the lower right corner of the figure explaining the different bar colors). Main findings are the well-known high SOX2 expression in EC and low expression in seminoma/dysgerminoma. The profile of SOX17 shows an inverse relation with low expression in EC and high expression in seminoma/dysgerminoma samples and the seminoma cell line TCam-2 (indicated by an asterix).

seminoma/dysgerminoma cells, being therefore co-expressed with POU5F1 (Figure 3B and 3C). In addition, the seminoma cell line TCam-2 – expresses SOX17 protein (Figures 3D and 3E), while no SOX17 was detected on cytospins of EC cell lines (data not shown) and by Western blot analysis (Figure 3E). The specific nuclear staining for SOX17 can be of diagnostic value in discriminating seminoma/dysgerminoma from EC. However, SOX17 is not a suitable marker for the diagnosis of CIS, because it is found in different maturation stages of spermatogenesis (Figure 3F). In addition, SOX17 expression is present in fetal male and female gonocytes (earliest sample tested was 57 days old gonad) and stays present in these cells throughout fetal life (Figure 3G). No staining was identified in either Sertoli cells or granulosa cells. In contrast to male spermatogenesis, SOX17 is not detected in oogenesis beyond the stage of the gonocyte. However, SOX17 protein is detected in the epithelium of the Fallopian tube and rete testis (data not shown). Therefore POU5F1 is still the best marker for detection of CIS in the adult testis [32].

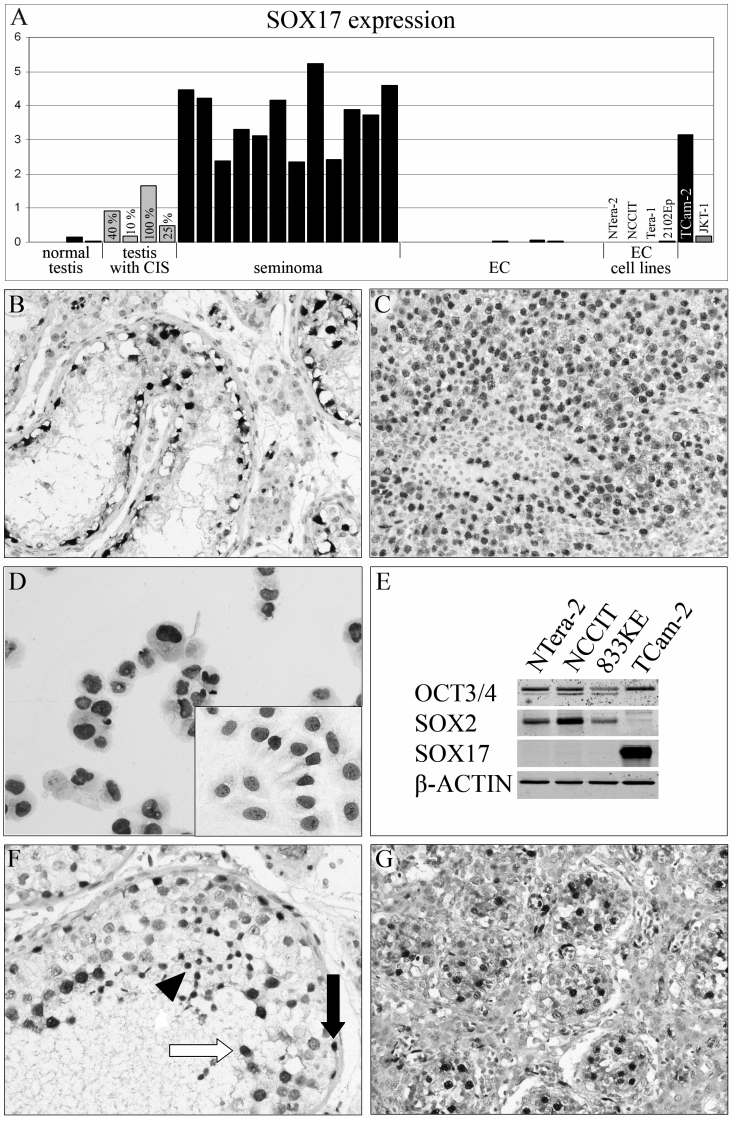

FIGURE 3. A) Quantitative RT-PCR for SOX17 on TGCT samples, cell line JKT-1, seminomatous cell line TCam-2 and EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep) demonstrates a high expression of SOX17 in the seminoma samples and cell line TCam-2, whereas the expression is low in all other samples. Nuclear SOX17 signal (brown) in B) CIS, C) seminoma and D) the seminomatous cell line TCam-2. Inset: nuclear POU5F1 staining in TCam-2 cells. E) Western blotting shows a complementary expression pattern for SOX2 and SOX17 in TCam-2 and EC cell lines with high SOX17 expression in TCam-2 and no SOX17 expression in the EC cell lines NTera-2, NCCIT and 833KE. SOX2 is highly expressed in the EC cell lines and not in TCam-2. The embryonic stem cell marker OCT3/4 is expressed in all samples. As a loading control β-actin was used. F) SOX17 protein is present in various maturation stages of spermatogenesis: spermatogonium (black arrow), secondary spermatocyte (white arrow) and spermatid (black arrowhead), while primary spermatocytes, spermatozoa and Sertoli cells are negative. G) Nuclear SOX17 in gonocytes of an 18 week old fetus. *(Figure 3 colour image on page 189)*

DISCUSSION

Transcription factors act as pathway switches in embryonic development and commitment to the differentiation lineages. Specific combinations of transcription factors belonging to the SOX and POU families are known to establish tissue and cell specific expression of target genes. In this paper we focused on the protein partners of POU5F1 which are subsequently expressed during embryonic development in cells of the inner cell mass, the epiblast and PGCs. In ES cells the intimate relationship between POU5F1 and SOX2 is most extensively studied and binding of both proteins to adjacent sites on the DNA with simultaneous physical protein interaction controls a large number of target genes involved in pluripotency and differentiation.

Besides ES cells, SOX2 is abundantly expressed in differentiating and differentiated cells and is found in endodermal cells of the primitive foregut [50], the developing inner ear [51] and the central nervous system [52]. However, it is never detected in combination with POU5F1, which is likely related to the absence of pluripotency. It has been reported that *SOX2* is expressed in mouse fetal germ cells and neonatal ovine gonads [53, 54], but the actual data were not shown. These reports were confirmed by RNA *in situ* detecting SOX2 in male E12.5 to E15.5 gonad/mesonephros tissues, however no protein detection was done [55]. Recently, the expression of SOX2 in the establishment and early migration of mouse PGCs was identified by quantitative single-cell gene expression profiling [56]. These results indicated that SOX2 expression is not immediately lost upon commitment to the germ cell lineage. However, the timing of SOX2 downregulation in gametogenesis remains to be clarified. Our results show that SOX2 is not expressed in human gonadogenesis from 15 weeks of gestation onwards, as well as in normal adult testis and ovary.

The study in early mouse PGCs also investigated the expression pattern of *SOX3* and *SOX17* [56]. Both genes were transiently expressed in migrating PGCs at developmental stage E7.25 dpc. Here we demonstrated for the first time that *SOX17* is expressed and the protein is present in CIS and seminoma cells, and not in EC cells, either *in vivo* tumors or cell lines. The absence of *SOX3* expression in seminoma (Figure 2) could be related to the timing of the maturation block that has occurred in the precursor cells of TGCTs, or the result of species specific differences. So far, the existing evidence for a role of SOX17 in gametogenesis is fragmented. Heterozygous *SOX17* knock-out mice are normal and fertile, while homozygous knock-out mice are deficient of gut endoderm and after 10.5 dpc no homozygous mutants are found [57]. This lethality due to endoderm deficiency is prior to gonad development and therefore the role of SOX17 absence during gametogenesis can only be studied in conditional knock-outs. Furthermore, two isoforms (1 without HMG-domain) have been isolated from an adult mouse testis library. Analysis of these isoforms suggest that SOX17 may function as a transcriptional activator in pre-meiotic germ cells, and that a splicing switch into a truncated SOX17 protein may lead to loss of its function in post-meiotic germ cells [35]. So far, no human SOX17 splice variants have been identified. The co-expression of SOX17 and POU5F1 in CIS and seminoma cells in the absence of SOX2, qualifies SOX17 as a potential protein partner in these cells. In addition, *SOX17* is a repressed target gene of the POU5F1 protein and not of SOX2 in human ES cells [22]. It remains to be investigated whether this is also the case in PGC. The co-expression of

SOX17 and POU5F1 in the seminoma cell line TCam-2 compared to established EC cell lines, being SOX2 positive and SOX17 negative, can be used as an experimental model to study this hypothesis, and to identify common target genes in CIS and seminoma. Possibly factors of the WNT pathway are involved in the transcriptional activity of POU5F1 and SOX17 in normal and malignant germ cells, as it has been shown in ES cells[58] and embryonic endoderm[59] that the proteins can physically interact with β-CATENIN. A recent observation is that SOX17 is essential for fetal and neonatal hematopoietic stem cells (HSC), but not for adult HSCs [37]. Knock-out studies and expression data of SOX17 in primitive endoderm, fetal HSCs and early germ cells indicates an crucial role of this transcription factor in a number of lineages during embryonic and fetal development. The origin of PGCs from the primitive endoderm, like fetal HSC, is of high interest in this context.

As suggested, the presence of expression of SOX17 in CIS could indicate the timing of the first step in the development of TGCTs. Whether these cells are already malignantly transformed at the time of this maturation arrest, or acquire malignant potential later in development as a result of an unfavorable micro-environment for immature germ cells is not clear. Therefore, analysis of these markers in patients with Disorders of Sex Development (DSD) will be informative, because some of them show a significant increased risk for type II GCTs [30]. Our study of SOX2 expression in normal gonadogenesis and TGCTs demonstrated that SOX2 can be present in Sertoli cells in CIS-containing seminiferous tubules, whereas this was rarely observed in tubules without CIS. SOX2 is not expressed in fetal gonads from 15 weeks of gestation onwards, indicating either aberrant expression of SOX2 in Sertoli cells in CIS-positive tubules, or possibly persistent expression assuming SOX2 is expressed in Sertoli cells before 15 weeks. Either way, SOX2 expression in CIS-positive tubules reflects an abnormal micro-environment which may have contributed to the initiation of TGCT development or has a later origin under the influence of the aberrant germ cells. An important implication of this result is that SOX2 expression in seminiferous tubules should not be mistaken for intratubular EC known to be SOX2 positive.

The differential and complementary expression of SOX2 and SOX17 in normal and malignant germ cell development and identification of SOX17 as the alternative protein partner for POU5F1 in CIS and seminoma cells provides an important lead to elucidate the cell specific function of POU5F1 in embryonic germ and ES cells. The recently characterized seminoma cell line TCam-2 can be used to study the mechanisms and identify the involved target genes of joined POU5F1 and SOX17 action.

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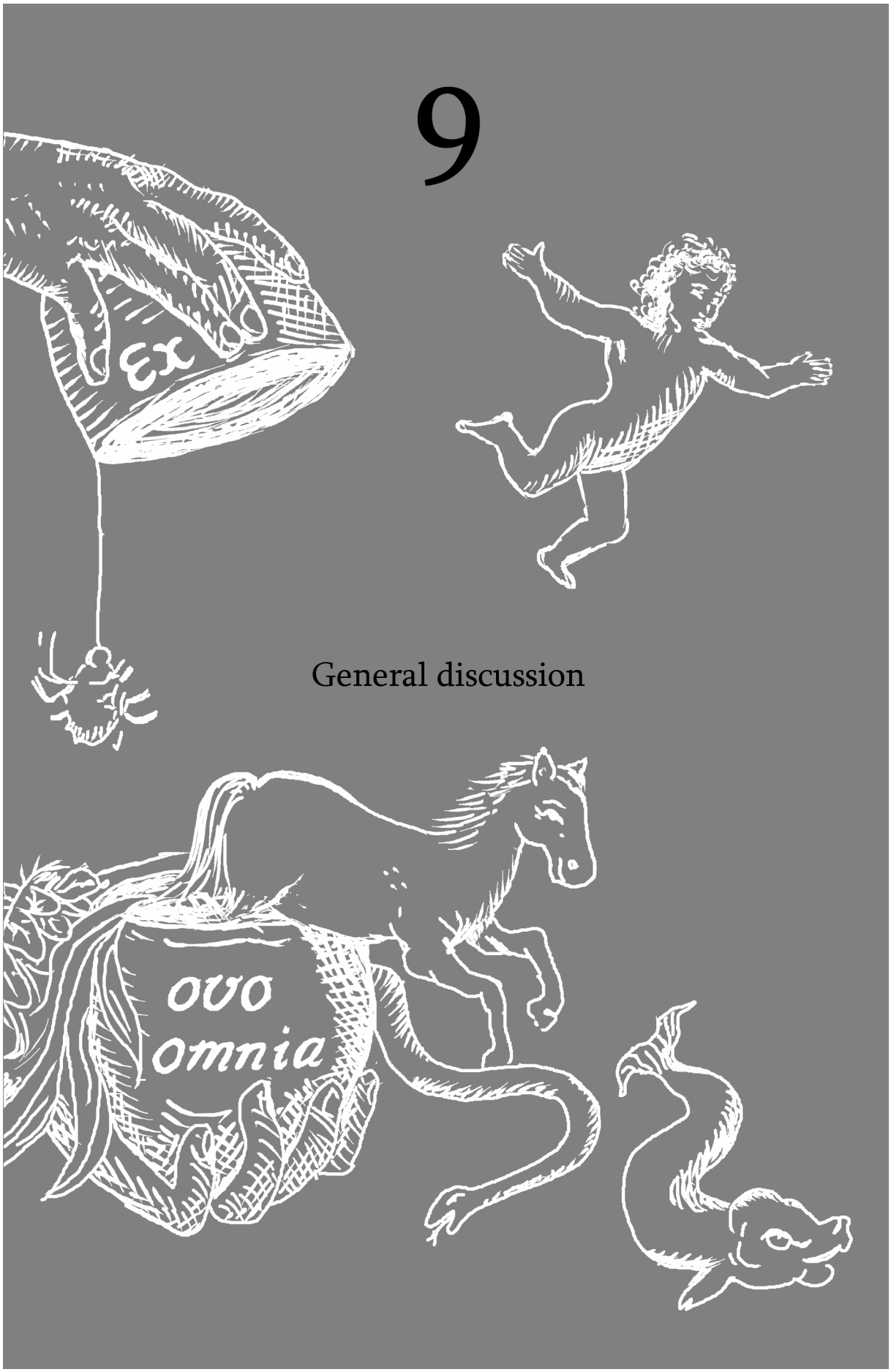

General discussion

GENERAL DISCUSSION

The pluripotency of the first stages of embryogenesis is inherited by the future generation via the gametes. Paradoxically, in order to transmit pluripotency, the gametes have to give up their own pluripotent potential and have to differentiate along the path of spermatogenesis or oogenesis including meiosis, which reduces their chromosome content from diploid to haploid. During this initial maturation process embryonic markers involved in pluripotency are progressively downregulated reflecting loss of pluripotent potential. In contrast, germ cells that retain their embryonic features will interfere with fertility and can acquire malignant potential culminating in a germ cell tumor. The expression of embryonic markers in these tumors can be used as a powerful diagnostic tool in clinical pathology. The transcription factor OCT3/4 is currently regarded as one of the key regulators of pluripotency, and has been intensively studied in the research fields of stem cells and germ cell tumors.

A POUERFUL DIAGNOSTIC MARKER IN TYPE II GERM CELL TUMORS

Expression of OCT3/4 was initially found in a mouse embryonal carcinoma (EC) cell line[1], and shortly after that in early stem cells and germ cells of the mouse embryo[2-5]. In human testicular GCTs the presence of OCT3/4 mRNA was reported in 2002[6], and a year later an extensive study on OCT3/4 protein expression in a multi-tumor tissue micro-array analysis was published[7]. This immunohistochemical study using a goat polyclonal antibody showed that OCT3/4 is specifically expressed in CIS/gonadoblastoma, seminomatous and EC cells of type II GCTs and not in other tumors. These findings were confirmed by a large number of other studies on samples collected from various archives[8-15]. The first prospective study on the value of OCT3/4 as a diagnostic marker in TGCTs was done on a consecutive series of more than 200 testicular cancers (Chapter 2)[16]. Next to the polyclonal antibody used in all previous studies, the value of a mouse monoclonal antibody was demonstrated. This monoclonal antibody gave comparable results to the polyclonal antibody in immunohistochemistry and Western blotting. Both antibodies were shown to be robust and highly stable, and the immunohistochemical detection method applied was relatively insensitive to pretreatment or quality of the tissue samples. The strong and reproducible nuclear staining of OCT3/4 was present in 100% of CIS, seminoma and EC cells. This study demonstrated that OCT3/4 is a valuable and reliable marker in the diagnostics of TGCTs. However, evaluation of immunohistochemistry should always be performed in the context of cell morphology and proper controls. This is exemplified by subsequent studies on the expression pattern of OCT3/4 in metastatic EC and seminoma. Sung *et al* [17] reported on OCT3/4 staining of metastatic EC after chemotherapy. Six out of 25 cases (24%) were negative for both CD30 and OCT3/4. This unexpected result was proven to be erroneous, because five out of six cases were identified as yolk sac tumors based on a positive AFP staining (reviewed in Cheng *et al* [18] and own unpublished results) and the remaining case was a non-germ cell malignancy judged by morphology. In other words, all EC, even after chemotherapy, are proven to be OCT3/4

positive. In seminoma metastases Biermann *et al* [19] found a very heterogeneous staining of seminoma cells using OCT3/4 immunohistochemistry. The percentage of OCT3/4 positive tumor cells ranged from 5 to over 90%. In addition, 10 cases of testicular seminoma also showed a major variation from 25% to over 90%. Because of the discordance of these results with previous findings, immunohistochemistry for OCT3/4 was performed by the same group with a new batch of monoclonal antibodies and adjusted protocol, as used in our study.[16] The results showed a consistent, intense and homogeneous staining of all tumor cells. Therefore, the initial results must be due to sub-optimal staining conditions. This finding is in preparation to be submitted for publication (Biermann and coworkers). In diagnostic challenging cases we have shown that OCT3/4 can be crucial to come to a final diagnosis (Chapter 2 and 3). Especially when other markers as PLAP and c-KIT are negative, small biopsies having a limited number of neoplastic cells are present and tissue samples of poor quality due to necrosis, the consistent strong nuclear staining of OCT3/4 can be the decisive diagnostic clue.

OCT3/4 is not only a valuable marker in diagnosis of overt TGCTs, but can also significantly contribute in screening for occult tumors and their precursor cells (Chapter 2 and 4). In a series of 81 open surgical testicular biopsies from 56 adults with primary infertility or a history of TGCT, CIS cells were identified in four patients (7%) based on morphology and OCT3/4 immunohistochemistry[16]. Of importance is our observation of the occasional presence of OCT3/4 positive cells lacking typical CIS morphology in seminiferous tubules that contain normal spermatogenesis through all maturation stages. Because the normal adult testis including spermatogenesis is OCT3/4 negative and these normal appearing tubules were adjacent to tubules with typical CIS cells and without spermatogenesis, the OCT3/4 positive cells in normal seminiferous tubules were diagnosed as CIS. These reports and the difficulty to morphologically identify all CIS cells based on formalin-fixed tissue demonstrate that screening for OCT3/4 positive cells in populations of patient with risk factors for TGCTs, like infertility, cryptorchidism and a (familial) history of TGCT, is mandatory when these patients are biopsied (Chapter 4). Chances of finding CIS or an early occult tumor are significantly raised in comparison to the general population in which the incidence is 6-11 per 100.000. In nation-wide search of invasive TGCTs with a previous negative testicular biopsy, obtained because of infertility studies, we could prove the presence of CIS cells by positive OCT3/4 immunohistochemistry in 10 out of 21 cases.

MULTIPLE OCT3/4 PSEUDOGENES ARE TRANSCRIBED

Several reports on OCT3/4 protein expression in differentiated cells have been published.[20-22] A report on bladder cancer by Atlasi *et al* [23] demonstrated OCT3/4 mRNA expression using a non-specific primer set (see below) and in addition OCT3/4 protein expression by immunohistochemistry and Western blotting using both OCT3/4 antibodies that were validated by us [16]. The Western blot images shown for both antibodies appeared genuine and specific, although the signal in the bladder sample lanes had a slightly larger mass than the NTera-2 control lane. These results could not be confirmed in a series of

bladder cancers of our own institute (unpublished results). The immunohistochemistry in the report by Atlasi and coworkers provide no clear images of evident nuclear OCT3/4 staining, and they even show cytoplasmic staining which is incompatible with functional OCT3/4. In most other report on OCT3/4 immunohistochemistry in differentiated normal or tumor cells, the presumably OCT3/4 positive nucleus is morphologically not well defined nor visibly surrounded by a cell. Therefore, we regard these staining spots as non-specific artefacts in contrast to the specific nuclear staining in ES cells, PGCs, and their malignant counterparts.

TABLE 1 Chromosomal location and name of human *OCT3/4 (POU5F1)* pseudogenes*

Chromosomal location	Approved gene symbol	Alias	Name in Pain *et al* [24]	Name in Suo *et al* [25]
1q22	POU5F1 pseudogene	LOC645682	Pg4	Pg3
3q21**	-	-	Pg6	Pg4 (Pg5 in table)
8q22	-	-	Pg2	Pg6
8q24	POU5F1P1	OTF3C; OTF3P1; POU5FLC8	Pg1	Pg1
10q21	-	-	Pg5	Pg5 (Pg4 in table)
12p13	POU5F1L	OTF3L	Pg3	Pg2

*Not all pseudogenes have an official NCBI name. In addition, the articles reporting the pseudogenes assigned different names to the pseudogenes. The article from Suo *et al* [25] uses inconsistent pseudogene names in text and table.

**The pseudogene on chromosome 3q21 is the only non-processed pseudogene, the others are processed and therefore intron-less pseudogenes.

Besides OCT3/4 protein expression, an alarming number of articles on OCT3/4 mRNA expression in a wide variety of cells have been published during the last few years, especially in the haunt for adult stem cell and cancer stem cell markers. However, reports from OCT3/4 expression in normal adult tissues but also in cancer have to be interpreted with caution because of the existence of a number of *OCT3/4* pseudogenes. Pseudogenes are defined as genomic sequences with close similarities to the paralogous gene, either originating from duplication of the genomic sequence of the gene (non-processed pseudogene) or alternatively by insertion into the genome of double stranded sequence generated from single-stranded RNA (processed or retro-pseudogene).[26] These pseudogenes can be transcribed, but a protein will never be formed, however the functional relevance of these pseudogene transcripts is unclear. In the case of the *OCT3/4* gene, Takeda *et al* [27] identified one pseudogene as a retrotransposon on chromosome 8 and predicted at least four related sequences in the human genome. Now that the entire human genome has been sequenced, *OCT3/4* related sequences can be identified more easily. In 2005, Pain *et al* [24] reported on multiple pseudogenes from embryonic stem cell specific genes. Ten highly homologous pseudogenes for *NANOG*, 16 for *STELLAR/DPPA3* and six for *OCT3/4* (Table 1) were identified by nucleotide BLAST searches against the mRNA transcripts of the respective gene. One of the *OCT3/4* pseudogenes was localized near the *NANOG* and *STELLAR/DPPA3* gene on 12p13 and this region was found to be evolutionary conserved between human and mouse chromosome 6, possibly indicating a functional role of this pseudogene. In this analysis only the long splice variant (isoform A)

of the OCT3/4 mRNA was used. We repeated this query for both OCT3/4 transcripts and the OCT3/4 partner SOX2. The pseudogenes for OCT3/4 isoform A were confirmed, however no pseudogenes for isoform B and SOX2 were identified in the genome. The first and thus far only report on the expression of the pseudogenes in a panel of normal tissue, tumors and cell lines came from Suo *et al* [25]. In this study a screening for *OCT3/4* related transcripts was done by using universal primers which should detect transcripts from the *OCT3/4* gene as well as from all pseudogenes (NB: these primers do not detect the pseudogene on 8q22). Transcripts from the true *OCT3/4* gene on 6p21, from the pseudogene on 10q21 and from 8q24 were confirmed by sequencing cloned PCR products. No transcripts were identified in the normal tissues examined. However, some data with (pseudo)gene specific primers conflicted with the data from the universal primers. In addition, they showed that the primers used by Monk *et al* [28] detected transcripts in almost all tissues and cell lines tested, even including normal tissues. The study of Suo *et al* [25] demonstrates that previous and future results need to be interpreted with caution because of the existence of transcribed pseudogenes. The use of specific primers for PCR, followed by cloning and sequencing is mandatory. Both studies described above do not give the exact location and use different names for the pseudogenes. Not all pseudogenes have been exactly mapped and given an official name, and therefore the current status on position and name is presented in Table 1. For consistency and clarity we refer to the pseudogenes by their chromosomal location and recommend others to do so in future publications until official names have been assigned.

TABLE 2. Non-specific primer sets used in RT-PCR*.

	Pseudogenes detected						
Article	1q22	3q21	8q22	8q24	10q21	12p13	Tissue/cells in which OCT3/4 expression was found
Takeda[27]	+			+		+	heart, kidney, liver, placenta, spleen, pancreatic islets
Jin[29]	+			+			primary breast carcinomas, breast carcinoma cell lines
Monk[28]				+		+	breast-, pancreas- and colon carcinoma cell line
Tai[22]				+		+	various normal and tumor cell lines, various normal tissues
Ezeh[30]	+			+			primary breast carcinomas, breast carcinoma cell lines, seminomas
Matthai[21]				+		+	endometrium
Raman[31]				+		+	normal and tumor renal tissue
Hoffmann[32]	+			+		+	primary prostate tumors and cell lines, prostate and testis
Mongan[33]				+		+	cultured human epidermal keratinocytes

*These primers will detect not only OCT3/4 (POU5F1) mRNA but also transcribed OCT3/4 pseudogenes and genomic sequences of intron-less pseudogenes. This results in a large number of reports claiming OCT3/4 expression in normal and neoplastic differentiated tissues and cell lines.
We designed a primer set that is specific for mRNA of the true *OCT3/4* gene: F 5'-tcccttcgcaagccctcat-3' and R 5'-tgatgtcctgggactcctc-3'.

All studies so far have used non-specific primers that detect transcripts from the *OCT3/4* gene as well as from pseudogenes and this can be a major reason for finding expression of OCT3/4 in a broad gamut of cells and tissues. In addition, because five *OCT3/4* pseudogenes are processed intron-less pseudogenes, DNA contamination will give a false positive result. A selection of studies is presented in Table 2 to illustrate the non-

specificity of primers, the sequence of a specific primer pair we designed is added in the legend. A worrying finding is that probe sets used in micro array expression profiling like Affymetrix are not specific for the true gene but also detect some of the pseudogenes.

EPIGENETIC REGULATION OF THE *OCT3/4* GENE

As stated above, the true *OCT3/4* gene is only expressed in stem cells, germ cells and their malignant counterparts, i.e. EC and CIS/seminoma. During normal development the promoter region of *OCT3/4* undergoes significant methylation and this process has been extensively studied *in vitro* by differentiation of human and mouse ES and EC cells[34-37]. The progressive methylation of *OCT3/4* and *NANOG* sequences has recently been proposed as barometers to determine the extent of human ES cell differeniation[38]. We showed that the methylation status of patient-derived TGCTs of various histologies corresponds to the *in vitro* obtained results (Chapter 5). Differentiated tumor components as yolk sac tumor and teratoma had heavily methylated upstream regions of the *OCT3/4* gene, whereas samples primarily composed of seminoma or EC cells had a considerably lower level of methylation. In addition, micro-dissection of seminoma and EC cells showed the virtual absence of methylation. This is in agreement with the difference in overall methylation status of these various histological tumor types, likely reflecting normal development [39].
The developmental dynamics of shut down of *OCT3/4* expression follow a multi-step program of inactivation[40]. Binding of GCNF to the RA receptor element (RARE) causes transient transcriptional repression. This causes recruitment of histone deacetylase (HDAC) molecules and subsequently histone methylases. Finally, DNA methyltransferases DNMT3A and perhaps DNMT3B catalyse the *de novo* methylation of the *OCT3/4* regulatory sequences. Under normal circumstances this last step appears to be irreversible[40], but artificial removal of methylation by demethylating agent 5-azacytidine was shown to revert differentiation of mouse ES cells *in vitro* [41]. Dedifferentiation of cells is particularly of interest in the current attempts to generate cells with a stem cell phenotype from terminal differentiated somatic cells. The ultimate goal is to create autologous stem cells that can be used to replace injured or damaged tissues in patients suffering from different diseases, e.g., degenerative neurological diseases, diabetes, as well as ischemic heart disease[42-44].

DEDIFFERENTIATION, REPROGRAMMING AND CELL LINE MODELS

The first hurdles along this pathway has been taken by showing that forced re-expression of a small set of genes is sufficient to create cells with a stem cell phenotype, so-called induced pluripotent stem (iPS) cells[45]. Induction of pluripotency was achieved in adult mouse fibroblasts by retroviral introduction of transgenes expressing OCT3/4, SOX2, c-MYC and KLF4. The dominant role of OCT3/4 and SOX2 in pluripotency is well established, while c-MYC and KLF4 are known to contribute to rapid proliferation of ES cells in culture[46,47] and are frequently upregulated in tumors. Recently, viable and fertile

chimaeras were obtained by injection of iPS cells into blastocysts[48,49]. However, in one study approximately 20% of the offspring developed tumors attributable to reactivation of the c-MYC transgene. This negative side-effect is a major concern in the clinical application of iPS cells.

Dedifferentiation of cells into undifferentiated cells with an embryonic phenotype that are pluripotent and can differentiate into all cell lineages is exactly what happens in TGCTs *in vivo*. CIS and seminoma cells, the malignant counterparts of PGC/gonocytes, are blocked in the process of gametogenesis and have limited differentiation capacity. In seminomatous tumors hCG positive trophoblastic giant cells can be present which suggests that these cells originated from a seminoma cell. In almost half of TGCTs, CIS and seminoma cells can acquire the expression of ES cell markers and become pluripotent EC cells by a process called reprogramming. The trigger for this dedifferentiation and underlying molecular mechanisms are still unknown.

To study the process of reprogramming, an *in vitro* model that allows controlled manipulation of CIS or seminoma cells would be valuable. During the last two decades, considerable effort has been made to establish seminoma cell lines. However, seminoma cells rapidly undergo programmed cell death (apoptosis) upon disruption of their micro-environment and can only be cultured for a short period of time. In contrast to the large number of EC cell lines, there are only single reports on seminomatous cell lines[50-52]. One of these cells lines, JKT-1, described to originate from a testicular seminoma of a 40-year-old Japanese male, has been used for various investigations consistently as representative for seminoma[53-58]. We showed that JKT-1 does not have the essential hallmarks of a seminoma, like gain of 12p sequences and expression of OCT3/4 and NANOG amongst others, and therefore cannot be used as a model for seminoma[59] (Chapter 6). In addition, it was demonstrated that the tumor of which the cell line was supposed to be generated from was genetically unrelated. This indicates that a thoroughful characterization of a cell line is required before it can be accepted as informative. Another cell line, TCam-2, was reported in a Japanese paper to be established from a testicular seminoma of a 35-year-old Japanese male by the traditional explant culture method[51]. In an extensive characterization of this cell line, genomic constitution, imprinting status and immunohistochemical marker profile were determined, as well as genome wide mRNA and miRNA expression profiling (Chapter 7). The results showed that TCam-2 cells contain all the typical features of seminoma. In addition, an activating mutation of the *BRAF* gene, a downstream factor of the RAS pathway, was found, providing an explanation for successful establishment of this cell line.

One of the unique features of TGCT is that the initial steps in tumor development, already take place *in utero*. This makes it difficult to study the early steps in malignant transformation of the germ cells because of the inaccessibility of this cell population in the early embryo. During the past years, major progress has been made in establishing *in vitro* models for gametogenesis by generating PGCs from ES cells[60-63]. Recently, viable transgenic offspring was produced from sperm differentiated from mouse ES cells[64]. These results pave the way to a model system for the early phases of germ cell tumorigenesis.

Currently, the pre-invasive CIS cells are the earliest available stage for research in TGCT development, but there are no cell lines for CIS. After puberty, these cells start

proliferating, acquire additional chromosomal aberrations including gain of 12p, and become invasive, giving rise to a seminoma or – via reprogramming – to embryonal carcinoma[65]. Because seminoma is the default pathway, resembling CIS cells morphologically and expressing the same markers, the availability of the seminomatous cell line TCam-2 provides a model system for earlier steps in TGCT development than the EC cell lines.

PROTEIN PARTNERS AND MICRO-ENVIRONMENT

During normal development OCT3/4 is expressed in the inner cell mass, the epiblast, PGCs and gonocytes. The function OCT3/4 exerts is not the same in these cell types: in the inner cell mass/ES cells OCT3/4 expression will keep these cells in an undifferentiated pluripotent state, while in PGCs it is implicated in survival of these cells[66]. As OCT3/4 is a transcription factor, this differential function can be achieved by selection of cell specific target genes using cell specific co-factors. Sumoylation of OCT3/4 at the end of the N-terminal transactivation domain by SUMO-1 enhances the protein stability and increases DNA binding[67]. This mechanism is the first post-translational modification known to regulate OCT3/4 activity *in vitro* and *in vivo*. A limited number of proteins involved in selection of OCT3/4 target genes has been identified[68]. Another group of developmental regulators in early development are the polycombs. These proteins form multiple Polycomb Repressive Complexes (PRC) that can modify chromatin structure. After initial gene repression by transcription factors as OCT3/4, PRCs are brought to the site of initial repression and act through epigenetic modification of chromatin structure to maintain these genes in a repressed state[69]. The target genes of PRC2 in ES cells, become activated during ES cell differentiation when the repressive effect of PRC2 is released. The observation that OCT3/4, SOX2 and NANOG are bound to a significant subset of developmental genes occupied by PRC2 links this polycomb complex to the maintenance of stem cell pluripotency[70]. The molecular mechanism by which OCT3/4 and PRC2 are connected, remains to be elucidated.

In ES cells and also in EC cells, SOX2 is the well established protein partner for OCT3/4. SOX2 knock-out embryos are embryonic lethal[71] and the joined binding of OCT3/4 and SOX2 to enhancers of pluripotency genes was shown to be essential for maintaining pluripotency. However, a recent article[72] by the same group that reported on the role of the level of OCT3/4 expression in maintaining pluripotency[73] shows that SOX2 is redundant in regulating other pluripotency genes. Other SOX proteins present in ES cells like SOX 4, SOX11 and SOX15 can also bind to the enhancers of these genes and substitute for SOX2[72]. Indirectly, SOX2 is necessary for maintaining pluripotency by regulating multiple transcription factors that affect *OCT3/4* expression[72]. In TGCTs functional studies on the role of SOX2 are lacking. To characterize the SOX2 expression profile in TGCTs we performed immunohistochemistry on tissue micro-arrays of the various histological subtypes of TGCTs. CIS, seminoma, yolk sac tumor and choriocarcinoma were negative for SOX2; EC was positive and also some components of teratoma were positive as can be expected because SOX2 is expressed in various

differentiated tissues in normal development (Chapter 8). Surprising was the presence of SOX2 positive Sertoli cells in seminiferous tubules containing CIS. This phenomenon was shown to be aberrant SOX2 expression, as it was not present in normal Sertoli cell maturation during fetal and adult life. Whether SOX2 expression in Sertoli cells is a consequence of derailment of germ cell maturation in TGCTs or is inherent to an abnormal micro-environment in the seminiferous tubules that contributes to TGCT initiation is unknown. In search of an alternative protein partner for OCT3/4 in cells devoid of SOX2, we identified SOX17 as the candidate protein partner for OCT3/4 in gonocytes, CIS and seminoma based on quantitative PCR, mRNA expression profiling and immunohistochemistry on fetal gonads, clinical TGCT samples, seminomatous cell line TCam-2 and several EC cell lines (Chapter 8). The presence of SOX17 in seminoma and absence in EC indicates potential diagnostic value in discriminating seminoma from EC.

In ES cells SOX17 expression is repressed by joined action of OCT3/4[74] and PRC2[70]. The switch from SOX2 to SOX17 as a protein partner for OCT3/4 might be a lead to explaining the functional switch from keeper of pluripotency in undifferentiated cells to survival factor in PGCs. During migration of PGCs in the mouse embryo, there is a peak in SOX17 mRNA expression after which the expression ceases to virtually zero[75]. Our results show that in humans SOX17 protein is present from early fetal gonocytes to mature stages of spermatogenesis. In the female gonad SOX17 expression in only present in gonocytes and not in later stages of oogenesis. Of interest is the concurrent SOX3 peak in migrating mouse PGCs as SOX3 mRNA expression is not observed in clinical seminoma samples and the TCam-2 cell line. This initiates the question whether absence of SOX3 expression contributes to germ cell tumorigenesis? In favor of this hypothesis is the observation that absence of SOX3 expression in a mouse knock-out leads to impaired spermatogenesis and increased expression of OCT3/4[76].

ROLE IN ONCOGENESIS

Aberrant expression of OCT3/4 has been suggested to play an oncogenic role in tumorigenesis.[8,22,28] In order to act as an oncogene, OCT3/4 protein has to be present at least in the initial tumor cells. Thus far, OCT3/4 protein expression has only consistently and repeatedly been found in type II (T)GCTs.[7] However, it is highly questionable if the expression in these tumors can be regarded as aberrant. The cell of origin of these tumors is an embryonic PGC or gonocyte, which normally expresses OCT3/4.[65] In the invasive tumors, the seminoma and EC cells, which are the OCT3/4 positive cells in (T)GCTs, can be considered the malignant equivalents of respectively PGCs and ES cells as explained before. In addition, OCT3/4 expression is rapidly lost in type II (T)GCTs upon differentiation into teratoma along the three germ lineages of the embryo and into extra-embryonic tissues as yolk sac tumor and choriocarcinoma.[7] Based on these observations, OCT3/4 expression in (T)GCTs can be regarded as inherent to the embryonic phenotype of these cells, instead of the result of an activated oncogene with aberrant expression. Future studies need to address the presence of mutations or amplifications in the *OCT3/4* gene in GCTs.

The potential of OCT3/4 to act as an oncogene has been studied in several mouse models. Gidekel *et al* [8,77] injected mouse ES cells with various levels of induced OCT3/4 expression subcutaneously in nude mice. The highest percentage of tumor formation was seen after injection of ES cells expressing physiological levels of OCT3/4, whereas elevated (150%) or lower levels of expression (0, 50 or 75%) gave rise to less tumor formation. This indicates that non-physiological levels of OCT3/4 possibly impair cell proliferation or survival. In the tumors derived from OCT3/4 showing overexpression ES cells a significant higher percentage of primitive neuronal tissue was present, presumably indicating a higher grade of malignancy. However, from these results *OCT3/4* cannot be designated as a classical oncogene. In the same article, Gidekel and coworkers showed that mouse fibroblasts expressing transgenic OCT3/4 gave rise to morphological high-grade fibrosarcomas that can invade adjacent tissues after subcutaneous injection into nude mice. This indicates that ectopic expression of OCT3/4 can give a tumor-like phenotype. This hypothesis was tested by Hochedlinger *et al* [77] in a mouse model with inducible ubiquitously OCT3/4 expression in adult mice. Activation of *OCT3/4* resulted in dysplastic growths in epithelial tissues with an expansion of progenitor cells and a morphologically invasive phenotype. However, the lesions were dependent on continuous OCT3/4 expression and upon removal of this expression the dysplasia and even invasive growth disappeared completely. These studies illustrate that non-physiological expression of OCT3/4 can lead to dysplasia and even morphologically invasive behavior of cells in an artificial setup. However, OCT3/4 expression has never been consistently proven in human solid tumors other than (T)GCTs.

The only report possibly indicating a role of OCT3/4 in human non-GCTs is the identification of an *EWSR1-OCT3/4* fusion gene in an undifferentiated sarcoma derived from the pelvis bone of a 39-year old woman.[78] This fusion gene resulted from chromosomal translocation t(6;22)(p21;q12) creating a chimeric transcript composed of the transcriptional activation domain of the Ewing's sarcoma protein (*EWSR1/EWS*) gene and POU DNA-binding domain derived from *OCT3/4*. Although the functional relevance of the *EWSR1-OCT3/4* fusion gene is unclear as the presence of a fusion protein in the tumor cells was not examined, there is a strict relationship between tumor types and *EWSR1* fusion partners in Ewing's family tumors, suggesting a functional role of these *EWSR1* chimeras.[79] Of major interest is that EWSR1 is normally present in human and mouse ES cell lines where it co-activates transcription by binding directly to the OCT3/4 protein (Figure 1).[80]

The activity of the *OCT3/4* gene in type II TGCTs has been confirmed on DNA level as *OCT3/4* promoter hypomethylation together with expression of mRNA from the true gene and nuclear protein expression.[6,7,39] The presence of OCT3/4 and other stem cell markers in type II GCTs is likely to reflect the embryonic origin of these tumors instead of an initiating oncogenic event. In all other tumor types OCT3/4 expression still has to be consistently confirmed on the gene level, as well as on the mRNA- and protein level. Therefore, although OCT3/4 has oncogenic capacity in experimental systems, its role in oncogenesis of these tumors remains to be elucidated.

MAIN FINDINGS OF THIS THESIS AND FUTURE PERSPECTIVES

The results obtained in this thesis focus on the role of the transcription factor OCT3/4 in the development of TGCTs and provide a detailed analysis on the value of this factor in the diagnosis of these tumors. Based on a large series of consecutive tumors we conclude that OCT3/4 is a robust and reliable marker in type II TGCT diagnostics, by positive nuclear immunohistochemistry of all (100%) CIS, seminoma and EC cells. In challenging cases as poor tissue quality due to necrosis or a limited number of tumor cells in a histological biopsy, OCT3/4 immunohistochemistry was decisive in coming to a final diagnosis. Furthermore, a retrospective nation-wide analysis of invasive TGCTs with a previous negative testicular biopsy, showed the presence of CIS cells in these biopsies amid almost normal spermatogenesis. This finding exemplifies the need for OCT3/4 immunohistochemistry on testicular biopsies taken for infertility.
Germ cell tumorigenesis and embryogenesis parallel each other and their features can be compared to come to a better understanding of aberrant and normal aspects of the development of GCTs. We showed that the expression of *OCT3/4* in the various histological components of clinical TGCT samples is associated with specific changes in methylation of the promoter region and follows the same pattern as in embryogenesis. This adds to the view that OCT3/4 is expressed in TGCTs because it is inherent to its cell of origin, and not because of oncogenic activation. Analysis of the epigenetic state of the *OCT3/4* promoter, mRNA and protein expression was also used to validate whether the cell lines JKT-1 and TCam-2 could be used as a model for seminomatous tumors. Partly based on these results we showed that JKT-1 is not a seminomatous cell line, while TCam-2 is the first cell line that is available as a model for seminoma. In addition, we demonstrated the presence of an activating *BRAF* mutation in TCam-2, an exceptional event in *in vivo* TGCTs.
The relationship between OCT3/4 and its protein partner SOX2 has been extensively studied in ES and EC cells. We showed the presence of SOX2 positive Sertoli cells in seminiferous tubules containing CIS, the latter SOX2 negative. This knowledge is essential to prevent misdiagnosis of intratubular EC based on positive SOX2 staining. Furthermore, SOX17 is proposed as the alternative OCT3/4 protein partner in gonocytes, CIS and seminoma cells.

The findings presented above have generated new questions. In addition, longer existing questions can now be explored. The proven existence of a cell line representative for seminoma provides opportunities to mimic and study the process of reprogramming during TGCT development resulting in the clinically unfavorable non-seminomas. The switch from SOX17 as a potential OCT3/4 protein partner to SOX2 is likely to be essential during this transformation and this can be induced *in vitro*. In addition, target genes that are under joined SOX17 and OCT3/4 control can be identified in TCam-2. Recent progress in generating germ cells from ES cells has significantly enhanced the available research tools to investigate the earliest steps in germ cell tumorigenesis. Improved knowledge on the malignant transformation of the germ cell lineage is vital to mimic this process *in vitro* and eventually create an animal model for the type II GCTs.

Regarding diagnosis and treatment of TGCTs, future research should focus on an early diagnosis of these tumors as their precursor cells are already present from birth onwards. The long dormant phase at least till puberty should be taken advantage of to select and treat the carriers of the malignant cells before the tumors become invasive. OCT3/4 immunohistochemistry on testicular biopsy specimens will have to be implemented in clinical pathology routine. Screening tools for highly specific factors like OCT3/4 in blood and semen are being developed and will be applied to individuals at risk.
Pluripotency literally means to be able to do more. To beat the malignant germ cells, we have to increase our own pluripotency in diagnosis and treatment of these fascinating tumors.

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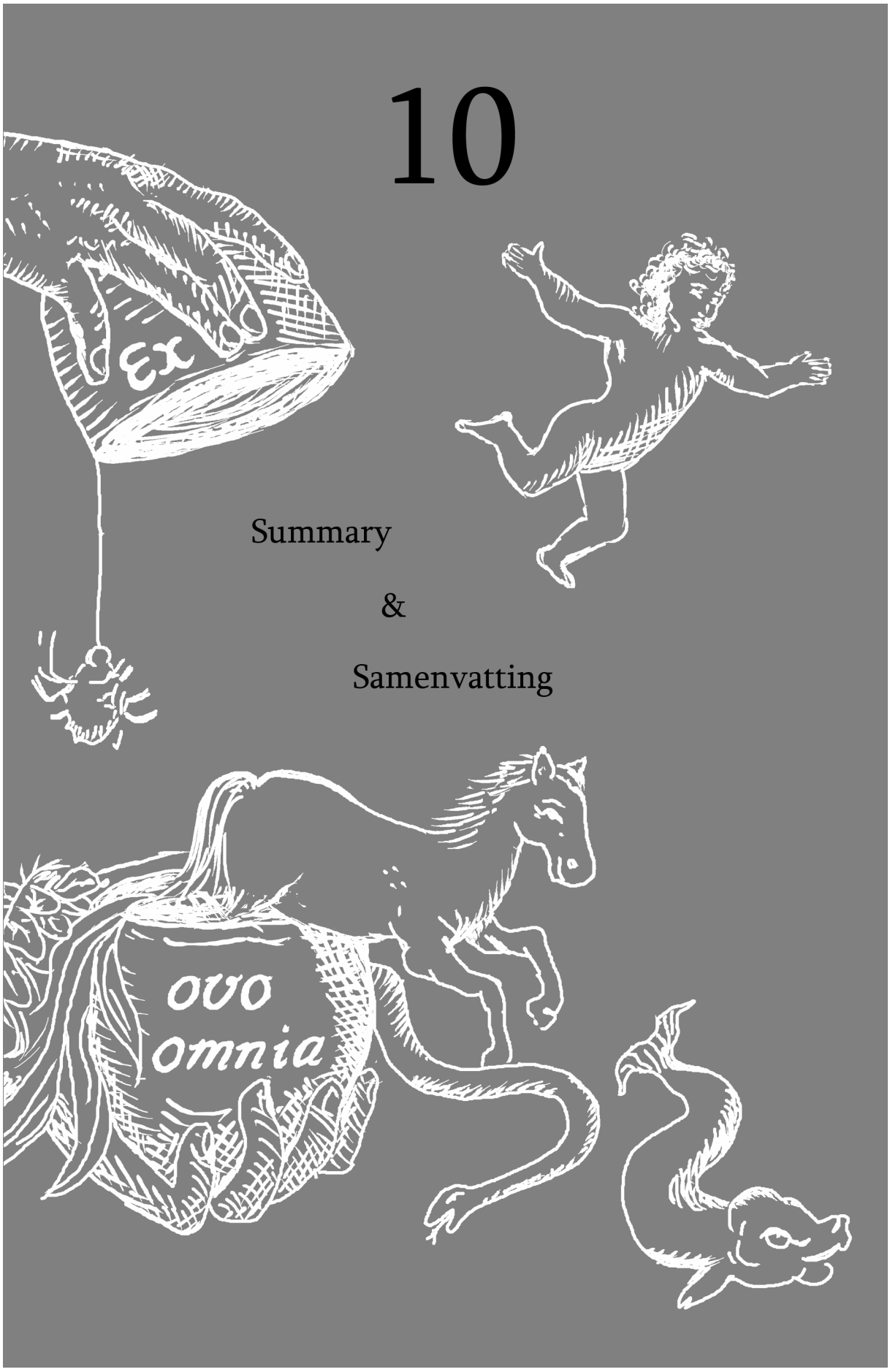

Summary

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Samenvatting

SUMMARY

Genesis of a new individual starts by fusion of the female egg and male sperm forming the fertilized egg called the zygote. This single cell will divide and the repeated division of its daughter cells will eventually give rise to approximately 100 million million cells that constitute an adult human being. Along this path of development the cells will acquire specialized features and skills necessary for performing their specific function within the organism; the cells have differentiated. If and how a cell will differentiate is dependent on the potency of the cell and the stimuli it receives from the environment. The first cell(s) of the embryo can differentiate to the complete gamut of somatic cells and germ cells, and are therefore pluripotent. The process of differentiation of germ cells starts in the first weeks of embryonal development and culminates in the formation of highly specialized germ cells, i.e. egg and spermatozoon, around puberty. Germ cells that fail to properly differentiate during development can give rise to germ cell tumors. Depending on the time point of derailment of normal germ cell development, different types of germ cell tumors can arise. This thesis focuses on germ cell tumors of adolescents and young adults that derive from an early germ cell that has not yet differentiated to a cell committed to male or female germ cell development. Reminiscent of their origin these tumors are pluripotent and have features that are only present in cells of early embryonic development. This includes the presence of proteins involved in regulation of pluripotency like OCT3/4, also known as POU5F1. Because OCT3/4 is unique to stem cells and germ cells in the early embryo and normally not present in the adult human, we can use OCT3/4 in diagnosis of germ cell tumors in adults.

Chapter 1 provides an extensive overview of the current knowledge of OCT3/4. The discovery of OCT3/4 and the recognition of its central role in the early steps of normal embryonic development as well as in germ cell tumor development is described. Furthermore, other genes and proteins involved in OCT3/4 regulation are grouped to provide a framework for future research.

In **chapter 2** the value of OCT3/4 in the diagnosis of a large consecutive series of germ cell tumors of the testis was examined. Two different antibodies to detect the OCT3/4 protein were used and both demonstrated the presence of OCT3/4 in the nucleus of specific tumor cell types. For clarity, a concise overview of the different cell types in germ cell tumors is provided here (see also figure 2 on page 20). Carcinoma *in situ*, the earliest known cell type of testicular germ cell tumors, is very similar to an early germ cell and has OCT3/4 protein in the nucleus of the cells. These cells are present in the seminiferous tubules where sperm is made from puberty onwards. When carcinoma *in situ* cells divide, become invasive and grow through the wall of the tubule they are referred to as seminoma cells. Fifty percent of all testicular germ cell tumors are composed of seminoma cells, which have also the OCT3/4 protein in their nucleus. The malignant germ cells - carcinoma *in situ* and seminoma - can transform by an unsolved mechanism (reprogramming) to a cell type that mimics the inner cells of the initial embryo. These malignant cells are called embryonal carcinoma, which is in principle pluripotent and can differentiate to all possible cell types of the embryo (teratoma tumor cells and malignant germ cells), placenta (the tumor cells are called choriocarcinoma), and yolk sac (yolk sac tumor). As

opposed to the seminomatous tumors (seminomas), tumors composed of embryonal carcinoma cells or any of their derived differentiated cells are referred to as nonseminomatous tumors (nonseminomas). The pluripotent potential of embryonal carcinoma is reflected by the presence of OCT3/4 in these cells. In the series of testicular germ cell tumors we examined, 100% of carcinoma *in situ*, seminoma and embryonal carcinoma cells were positive for the OCT3/4 protein, whereas all other cell types were negative.

In **chapter 3** the clinical history of a 40-year-old woman is presented who suffered from severe progressive neurological symptoms. On MRI scan a large diffuse area of aberrant intensity was seen. A stereotactic biopsy of this intracerebral lesion showed tumor cells on which no diagnosis could be made initially despite use of a large series of immunohistochemical markers. We showed that the neoplastic cells were positive for OCT3/4 and the final diagnosis was a diffusely infiltrating germinoma, which is a seminoma of the brain.

In **chapter 4** a retrospective study on patients who developed an invasive testicular germ cell tumor after an initial tumorcell negative biopsy is presented. Revision of the biopsies ans performing immunohistochemistry for OCT3/4 and other diagnostic markers revealed the presence of carcinoma *in situ* in ten out of 19 cases. This study shows that morphological examination is not sufficient and screening of all testicular biopsies by immunohistochemistry is mandatory to identify carcinoma *in situ*.

In **chapter 5** the methylation status of the upstream region of the *OCT3/4* gene in normal and neoplastic testicular tissues was examined. The cell line JKT-1, supposedly seminoma-derived, was included in this survey. We showed that all differentiated cell types, including JKT-1, had a consistent hypermethylation of the *OCT3/4* promoter. In contrast, seminoma and embryonal carcinoma were hypomethylated, confirmed by analyses after tumor micro-dissection. Our results from *in vivo* derived samples are in accordance to the previously generated *in vitro* data and show that expression of *OCT3/4* is associated with specific changes in methylation.

In **chapter 6** the origin of the cell line JKT-1 and its value as a model for germ cell tumors was investigated. This cell line was reported to be derived from a seminoma of a Japanese male. However, attempts to culture seminoma cells have failed in the past because these cells go into apoptosis (programmed cell death) after disruption of their micro-environment. We showed by analysis on chromosomal, DNA, RNA and protein level that JKT-1 lacks all the essential characteristics of seminoma cells and cannot be used as a model for these tumors. In addition, molecular analysis proved that the JKT-1 cell line and the supposedly primary tumor were not related.

Chapter 7 describes the characterization of another cell line, TCam-2, also reported to be derived from a seminoma of a Japanese male. The same approach as for the cell line JKT-1 was taken and all essential features of seminomas were present, including OCT3/4 mRNA and nuclear protein. A possible explanation for the successful establishment of this cell line was that TCam-2 had an activating mutation of the *BRAF* gene, a rare event in germ cell tumors.

In embryonic stem cells and embryonal carcinoma OCT3/4 exerts its function by physical interaction with SOX forming a heterodimer to activate and repress target genes. In these

undifferentiated cells OCT3/4 is regarded as the gatekeeper of pluripotency. However, in embryonic germ cells loss of OCT3/4 leads to programmed cell death instead of differentiation, implicating OCT3/4 as a survival factor in these cells. In **chapter 8** we show that SOX2 is not present in early fetal germ cells, carcinoma *in situ* and seminoma cells, and we propose SOX17 as an alternative protein partner for OCT3/4 in these cells. SOX17 mRNA and protein is present in patient derived seminoma samples and the seminoma cell line TCam-2, but not in embryonal carcinoma cell lines and patient samples. This indicates that SOX17 can be used as a diagnostic marker to differentiate between seminoma and embryonal carcinoma. Furthermore, SOX17 is present in early fetal germ cells, carcinoma *in situ*, and in almost all stages of spermatogenesis.
In **chapter 9** the findings of this thesis are discussed in a broader perspective and additional data are provided on the molecular biology of OCT3/4. In humans different variants of the OCT3/4 protein can be formed and there are several OCT3/4 pseudogenes, highly similar to the *OCT3/4* gene, but not suitable to make a protein. These *OCT3/4* pseudogenes and protein variants can hamper the interpretation of research results. Finally, we conclude that lack of OCT3/4 mutations and the presence of OCT3/4 and other stem cell markers in germ cell tumors are likely to reflect the embryonic origin of these tumors instead of an initiating oncogenic event.

SAMENVATTING

Het ontstaan van een nieuw organisme begint met de bevruchting waarbij een zaadcel de eicel binnendringt. Deze bevruchte eicel, de zygote, deelt zich herhaaldelijk en de gevormde dochtercellen vormen uiteindelijk de ongeveer 100 biljoen cellen waaruit een volwassen mens bestaat. Tijdens deze delingen krijgen de cellen speciale eigenschappen en taken die nodig zijn om hun specifieke functie in het lichaam te vervullen; de cellen zijn gedifferentieerd. Wanneer en op welke manier een cel differentieert hangt af van de cel zelf én van signalen uit de omgeving. De eerste cellen van het zich ontwikkelende embryo kunnen in elk denkbare andere lichaamscel differentiëren, bijvoorbeeld een spiercel, een zenuwcel of een rode bloedcel, en daarom worden deze eerste cellen pluripotent ("meer kunnend") genoemd. Ook de voorlopers van de geslachtscellen, genaamd kiemcellen, ontstaan uit deze pluripotente cellen tijdens de eerste weken van de embryonale ontwikkeling. Rond de puberteit rijpen de kiemcellen uit tot uiterst gespecialiseerde kiemcellen, de zaadcel en de eicel, waarvan de enige functie het vormen van een nieuwe generatie is. Kiemcellen met een verstoorde uitrijping kunnen zich ontwikkelen tot kiemceltumoren. Afhankelijk van het tijdstip waarop de normale kiemcelontwikkeling verstoord raakt, kunnen verschillende typen kiemceltumoren ontstaan. Dit proefschrift beperkt zich tot kiemceltumoren die zich vanaf de puberteit openbaren en voornamelijk bij jonge volwassenen voorkomen. Deze tumoren ontstaan uit vroege kiemcellen en hebben eigenschappen die normaal gesproken alleen bij cellen in het embryo voorkomen, zoals pluripotentie. Pluripotentie van een cel is afhankelijk van de aanwezigheid van bepaalde eiwitten, zoals OCT3/4, waarvan de officiële naam POU5F1 is. Dit eiwit speelt een sleutelrol bij de vroege ontwikkeling in het embryo. Het is bekend dat het aanwezig

blijven van dit eiwit in kiemcellen samenhangt met het ontstaan van kiemceltumoren. In het onderzoek dat beschreven wordt in dit proefschrift staat OCT3/4 en zijn rol in kiemceltumoren centraal. De onderzoeksresultaten vergroten onze kennis van kiemceltumoren en dragen bij aan optimalisering van diagnostiek waardoor de behandeling van patienten verder zal verbeteren.
In **hoofdstuk 1** wordt een uitgebreid overzicht gegeven van de huidige kennis over OCT3/4. De ontdekking van OCT3/4 en zijn centrale rol zowel in het begin van de normale embryonale ontwikkeling als in de ontwikkeling van kiemceltumoren wordt beschreven.
De waarde van OCT3/4 in het stellen van de diagnose kiemceltumor wordt beschreven in **hoofdstuk 2**. Hiervoor is gebruik gemaakt van kiemceltumoren die oorspronkelijk in een zaadbal ontstaan zijn. De aanwezigheid van het OCT3/4 eiwit in de kern van bepaalde tumorcellen kon worden aangetoond met twee specifieke antilichamen. Elk antilichaam herkent een ander gedeelte van het OCT3/4 eiwit. Beide antilichamen geven een duidelijk signaal in de celkern als het OCT3/4 eiwit aanwezig is. Voor een beter begrip van de verschillende celtypen in kiemceltumoren volgt hier een beknopt overzicht (zie ook figuur 2 op bladzijde 20). Het vroegst bekende celtype waaruit alle kiemceltumoren in de zaadbal van (jonge) mannen ontstaan is carcinoma *in situ*. Deze kwaadaardige kiemcellen lijken qua uiterlijk op vroege kiemcellen en hebben het OCT3/4 eiwit in hun celkern. Carcinoma *in situ* cellen liggen in de buizen van de zaadbal waar vanaf de puberteit de zaadcellen worden aangemaakt. Zodra carcinoma *in situ* cellen zich gaan delen en dwars door de wand van de zaadbuizen het omgevende weefsel binnendringen worden ze seminoomcellen genoemd. Van alle kiemceltumoren van (jong) volwassenen bestaat vijftig procent uit seminoomcellen die ook het OCT3/4 eiwit in hun celkern hebben. Kwaadaardige kiemcellen – carcinoma *in situ* en seminoom – kunnen veranderen in een cel die sterk lijkt op de eerste cellen van het embryo, de embryonale stamcellen. Dit veranderingsproces wordt reprogrammering genoemd, maar hoe dit verloopt, is nog een raadsel. De kwaadaardige embryonale stamcellen die via reprogrammering ontstaan worden embryonaal carcinoomcellen genoemd. Embryonaal carcinoom is pluripotent en heeft OCT3/4 eiwit in de celkern. Als het OCT3/4 eiwit verdwijnt, gaat embryonaal carcinoom differentiëren en kan dan veranderen in alle mogelijke celtypen in de mens en weefsels die om het embryo heenliggen tijdens de vroege ontwikkeling, zoals placenta (choriocarcinoom) en dooierzak (dooierzaktumor). Ondanks dat grotendeels het patroon van normale ontwikkeling gevolgd wordt, blijven deze cellen kwaadaardig. Als tegenhanger van de tumoren die uit seminoomcellen bestaan (seminomen), worden de tumoren van embryonaal carcinoom en daaruit ontstane cellen nonseminomen genoemd. In de grote verzameling kiemceltumoren van de zaadbal die geanalyseerd is, bevatten 100% van alle carcinoma *in situ* cellen, seminoomcellen en embryonaal carcinoomcellen het OCT3/4 eiwit. Alle andere celtypen missen het eiwit. Hieruit blijkt dat het aantonen van de aanwezigheid van OCT3/4 zeer geschikt is om kiemceltumoren te herkennen en daarom gebruikt kan worden door de patholoog om de diagnose kiemceltumor te stellen.
Hoofdstuk 3 beschrijft de ziektegeschiedenis van een vrouw van 40 jaar die verschillende neurologische symptomen kreeg waaronder wazig zien en vermindering van spierkracht en tastgevoel. Een MRI scan liet een onscherp afwijkend gebied in de hersenen zien en

hieruit werd een weefselbiopt genomen. In het weefsel waren tumorcellen aanwezig, maar het soort tumor kon niet vastgesteld worden, ondanks weefselkleuringen met een uitgebreid panel van specifieke antilichamen (immuunhistochemische kleuringen). Het nut van OCT3/4 voor de diagnostiek van kiemceltumoren was net bekend en uiteindelijk werd deze marker ook gebruikt op het weefselbiopt van de hersenafwijking. Alle tumorcellen waren positief voor OCT3/4 en de uiteindelijke diagnose was een diffuus groeiend germinoom, ook wel seminoom van de hersenen genoemd. De patiënte werd bestraald en haar lichamelijke klachten verminderden sterk door de behandeling.

In **hoofdstuk 4** is een studie gedaan naar patiënten die een kiemceltumor van de zaadbal kregen, hoewel er in een eerder afgenomen zaadbalbiopt geen tumorcellen gezien waren. Het opnieuw bekijken van de biopten en immuunhistochemische kleuring tegen het OCT3/4 eiwit toonde aan dat er in de biopten carcinoma *in situ* cellen aanwezig waren in tien van de negentien patiënten. Deze resultaten geven aan dat carcinoma *in situ* cellen niet altijd goed te herkennen zijn en dat OCT3/4 kleuring op alle zaadbalbiopten noodzakelijk is om een kiemceltumor uit te sluiten.

Hoofdstuk 5 beschrijft onderzoek naar het verband tussen de activiteit van het *OCT3/4* gen en de aanwezigheid van OCT3/4 eiwit in een (tumor)cel. Hieruit blijkt dat als er veel methylgroepen aan het DNA van het *OCT3/4* gen zitten, het *OCT3/4* gen uitstaat en er geen OCT3/4 eiwit gemaakt wordt. Dit is het geval in gedifferentieerde cellen. In tegenstelling tot gedifferentieerde cellen zitten er in seminoomcellen en embryonaal carcinoomcellen weinig methylgroepen aan het DNA van het *OCT3/4* gen en staat het gen aan waardoor OCT3/4 eiwit aangemaakt wordt. Het nieuwe van deze studie is dat in plaats van gekweekte tumorcellen (cellijnen), tumorweefsel gebruikt is van patiënten waardoor een directer inzicht wordt gekregen in de werkelijke situatie van de tumorcel in het menselijk lichaam.

Zoals eerder gezegd, bestaat vijftig procent van de kiemceltumoren uit de zaadbal uit seminomen. Van de nonseminomen zijn meerdere cellijnen gemaakt, die we in het laboratorium kunnen kweken en manipuleren om zo meer te weten te komen over de werkelijke tumoren. In het verleden zijn vele pogingen gedaan om ook seminoomcellen te kweken in het laboratorium en er een stabiele cellijn van te maken. Dit is echter nooit gelukt omdat deze cellen een zelfmoord programma (apoptose) aanschakelen als ze uit de tumor losgemaakt worden en vervolgens allemaal doodgaan. **Hoofdstuk 6** gaat over de cellijn JKT-1 die verondersteld werd afkomstig te zijn van een seminoom uit de zaadbal van een Japanse man. De afkomst van JKT-1 werd onderzocht en de eigenschappen van de JKT-1 cellen werden vergeleken met seminoomcellen uit tumorweefsel van patiënten. Uit analyse van de chromosomen, het DNA, RNA en eiwit van de JKT-1 cellen, blijkt dat een JKT-1 cel niet op een seminoomcel lijkt. Daarbij kon ook met DNA analyse worden aangetoond dat de JKT-1 cellen en het oorspronkelijke seminoom niet van dezelfde persoon afkomstig zijn. Deze resultaten laten zien dat de JKT-1 cellijn niet geschikt is als model voor het seminoom.

Hoofdstuk 7 is op dezelfde wijze opgezet als het voorgaande hoofdstuk. In hoofdstuk 7 is de cellijn TCam-2 onderzocht. Van deze cellijn is ook beschreven dat hij afkomstig is van een seminoom uit de zaadbal van een Japanse man. Uitgebreide analyse van TCam-2 cellen laat zien dat deze cellijn alle eigenschappen heeft die ook in seminoomcellen

aanwezig zijn, zoals OCT3/4 eiwit in de celkern. Hiermee is TCam-2 de eerste en – voor zover bekend – de enige cellijn die als model voor seminomen gebruikt kan worden. Een mogelijke verklaring voor het bestaan van deze unieke cellijn is dat TCam-2 cellen een mutatie in het DNA hebben op de plaats van het *BRAF* gen. Dit gen is vaak gemuteerd in verschillende soorten tumoren, maar zelden in kiemceltumoren.
De manier waarop het OCT3/4 eiwit zorgt dat cellen pluripotent blijven, is door binding op specifieke plaatsen in het DNA. Het OCT3/4 eiwit heeft hierbij hulp van andere eiwitten nodig, waarvan SOX2 het meest onderzocht is. In embryonale stamcellen en tumorcellen van embryonaal carcinoom binden het OCT3/4 eiwit en het SOX2 eiwit naast elkaar aan het DNA en wordt er ook een onderlinge verbinding gemaakt tussen de eiwitten. Hierdoor worden genen geactiveerd die nodig zijn voor pluripotentie, en genen uitgeschakeld die betrokken zijn bij differentiatie. In **hoofdstuk 8** wordt beschreven dat het SOX2 eiwit niet aanwezig is in vroege kiemcellen, carcinoma *in situ* en seminoom. Het OCT3/4 is wel aanwezig in deze cellen en heeft een partner nodig om zijn functie uit te oefenen. SOX17, een ander eiwit uit de SOX familie, is een zeer goede kandidaat hiervoor. Dit eiwit zit in de celkern van vroege kiemcellen, carcinoma *in situ* en seminoomcellen, zowel van tumoren als de cellijn TCam-2. Embryonaal carcinoomcellen bevatten geen SOX17 eiwit. Hierdoor kan SOX17 ook gebruikt worden om onderscheid te maken tussen seminoom en embryonaal carcinoom bij het stellen van een diagnose. Omdat SOX17 ook voorkomt in andere celtypen onder andere tijdens de uitrijping van de normale zaadcel, is het belangrijk om niet alleen naar de aanwezigheid van het SOX17 eiwit te kijken maar ook naar het uiterlijk van de cellen,
In **hoofdstuk 9** worden de resultaten van dit proefschrift besproken en vergeleken met wat bekend is in de huidige literatuur. Interessant daarbij is dat er in mensen verschillende varianten van het OCT3/4 eiwit bekend zijn, waarbij niet altijd duidelijk is wat de functie van zo'n eiwitvariant is én of dit wel een functie heeft. Naast het werkelijke *OCT3/4* gen in het DNA zijn er op een aantal chromosomen ook genen aanwezig die hier heel erg op lijken, pseudogenen genoemd, waarvan geen OCT3/4 eiwit gevormd kan worden. Door hun grote gelijkenis met het werkelijke gen kunnen ze voor verwarring zorgen.
De slotconclusie van het proefschrift is dat het OCT3/4 eiwit in kiemceltumoren een normaal eiwit is. Er zijn geen mutaties in het *OCT3/4* gen in kiemceltumoren bekend en de aanwezigheid van OCT3/4 verwijst naar de embryonale afkomst van deze tumoren.

CURRICULUM VITAE

The author of this thesis, Jeroen de Jong, was born on the 10th of September 1973 in the Holy Hospital in Vlaardingen. After obtaining his gymnasium-β diploma in 1992 at the Stedelijk Gymnasium in Schiedam, he commenced his academic carreer studying mathematics at the Leiden University. In 1993, he started his biology studies at the same university and explored his early interests in anatomy and morphology. A first training period at the Institute for Evolutionary and Ecological Sciences resulted in a thesis on morphology of the jaw musculature of the common swift. His first steps in molecular biology were taken in a study on the mouse *Orct3* gene at the Netherlands Cancer Institute in Amsterdam. In 1999, he obtained his Master of Science degree in the field of medical biology. The year before, he had started medical school at the Leiden University. In 2002, he graduated and started clinical work as a physician at Rijnaarde psychiatric hospital in Alphen aan den Rijn for a year.
A five month break in 2003 was spent in Peru and Bolivia where he learned the Spanish language and reached the summit of the Sajama vulcano at 6542 meters above sea level (photo on page 192). Back in the Netherlands he changed his focus from dissecting the mind to dissecting the human body. For half a year, he worked at the pathology department of the Medisch Centrum Haaglanden in Den Haag to gain expercience in macroscopy and autopsy. In 2004, he started his PhD research project on the role of OCT3/4 in germ cell tumors at the LEPO laboratory (Erasmus MC, Rotterdam) under the supervision of Leendert Looijenga and Wolter Oosterhuis which has culminated in this thesis.
Since October 2006, Jeroen is a pathology resident at the Erasmus MC in Rotterdam.

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Appendix of colour figures

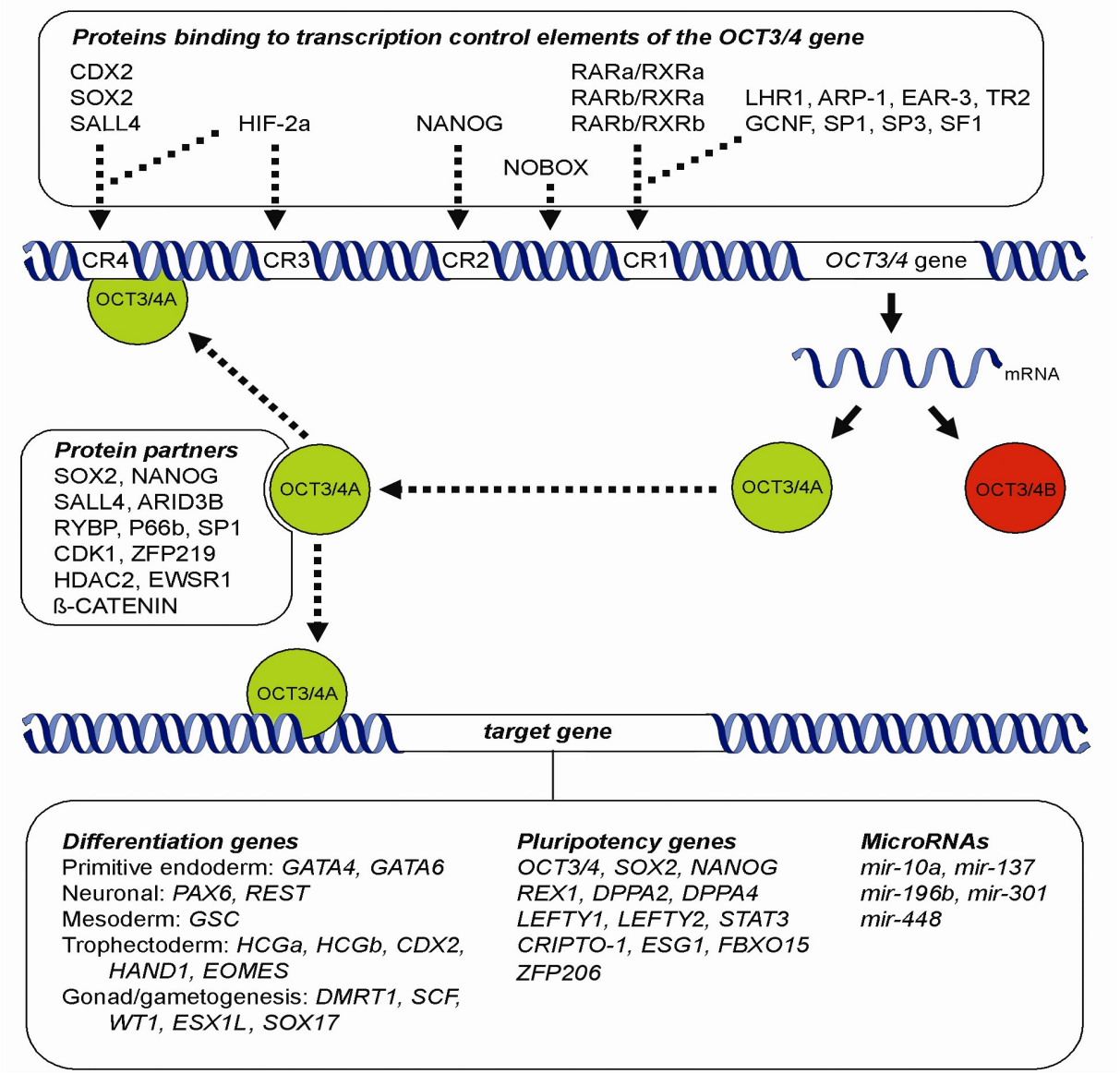

FIGURE 1. Transcriptional regulation of the *OCT3/4* gene and targets of OCT3/4 protein. For clarity the well-established name *OCT3/4* is used in this figure, however the official gene name is *POU5F1*. Transcription of the *OCT3/4* gene is regulated by a large number of proteins that bind to conserved regions (CR1 to CR4) in the upstream region of the gene. Which factors will bind depends on the stage of embryonic development, cell type and environment of the cells involved. The binding site for NOBOX (between CR1 and CR2) is only present in the murine *OCT3/4* gene, whereas, in theory, the other factors can bind in both the mouse and human gene. However, most of the functional studies have been performed in mouse and await confirmation in human cells. Activation of *OCT3/4* expression will generate mRNA that can be alternatively spliced in human generating two proteins, OCT3/4A and OCT3/4B. The biological role of OCT3/4B is unknown at the moment. The OCT3/4A protein can bind to several other proteins, but so far again most of these interactions have only been studied in mouse. In addition, OCT3/4A binds to transcriptional control elements and activates or represses expression of a large number of genes. Besides regulating its own expression by binding to conserved region four (CR4) upstream the *OCT3/4* gene, OCT3/4A regulates genes involved in differentiation and pluripotency of which a selected number from human and mouse data is presented here. Finally, OCT3/4A binds in the vicinity of a number of miRNAs and is therefore likely to influence their expression. *(Original position Figure 1 on page 16)*

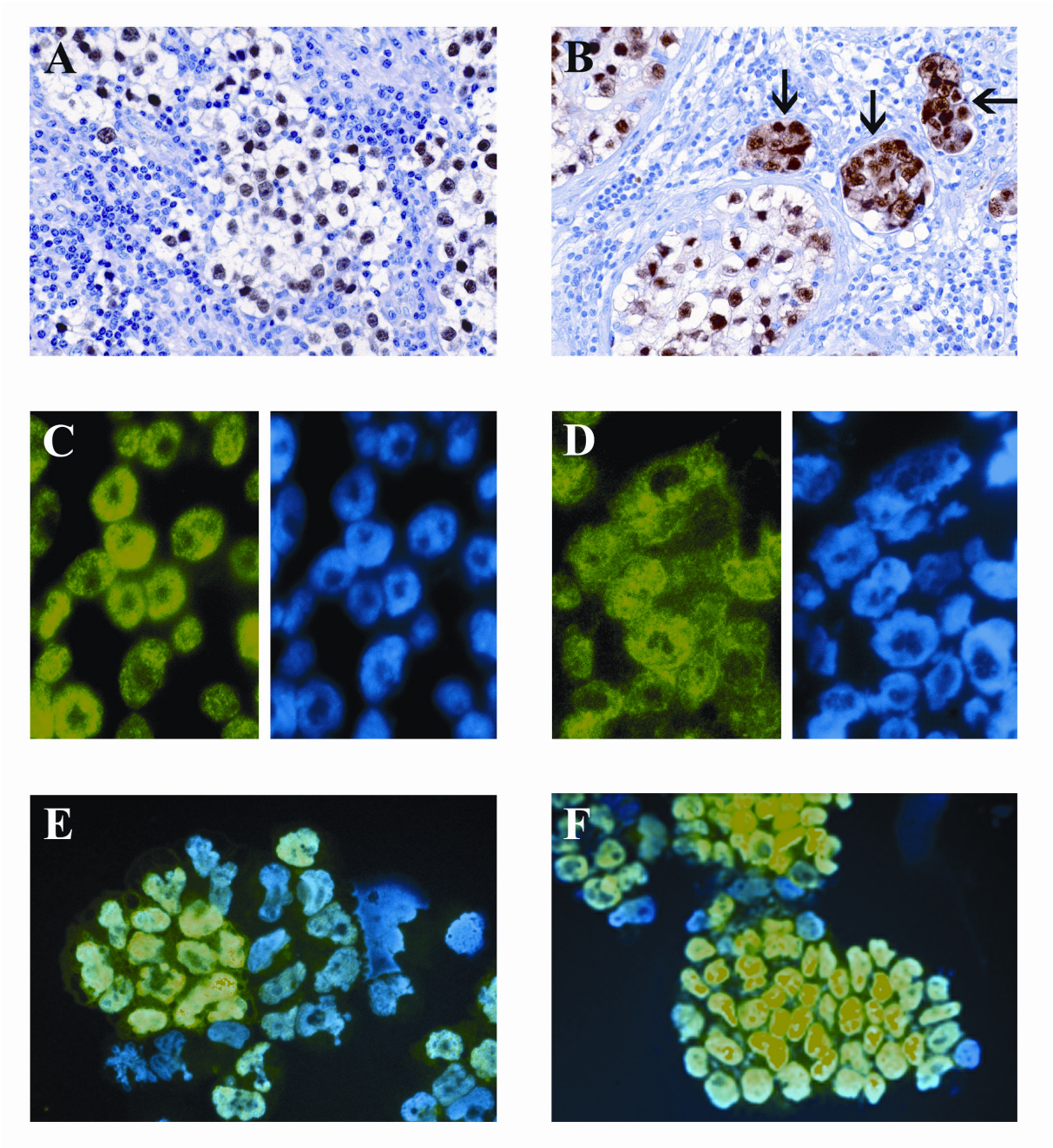

FIGURE 1. OCT3/4 immunohistochemistry using different embedding, fixation, and detection methods. (A) Nuclear staining (brown) of seminoma cells with a mono-clonal antibody. (B) Embryonal carcinoma and seminoma incubated with a polyclonal antibody. Note the cytoplasmic staining in the angio-invasive embryonal carcinoma (arrows) compared with the clear cytoplasm of intratubular seminoma cells. Both slides are from formalin-fixed and paraffin wax-embedded tissue. Images C–F show FITC-labelled fluorescence with the monoclonal antibody as the primary step. Nuclear staining is in blue (DAPI) and OCT3/4 localization in green (FITC). (C) Frozen tissue from a seminoma. (D) Frozen tissue from an embryonal carcinoma. Note the difference in cytoplasmic staining pattern between seminoma and embryonal carcinoma cells. Cytospin preparations of (E) pluripotent embryonal carcinoma-derived cell line NT2 and (F) nullipotent 2102Ep. Note the OCT3/4-negative cells located at the periphery of the aggregates.
(Original position Figure 1 on page 40)

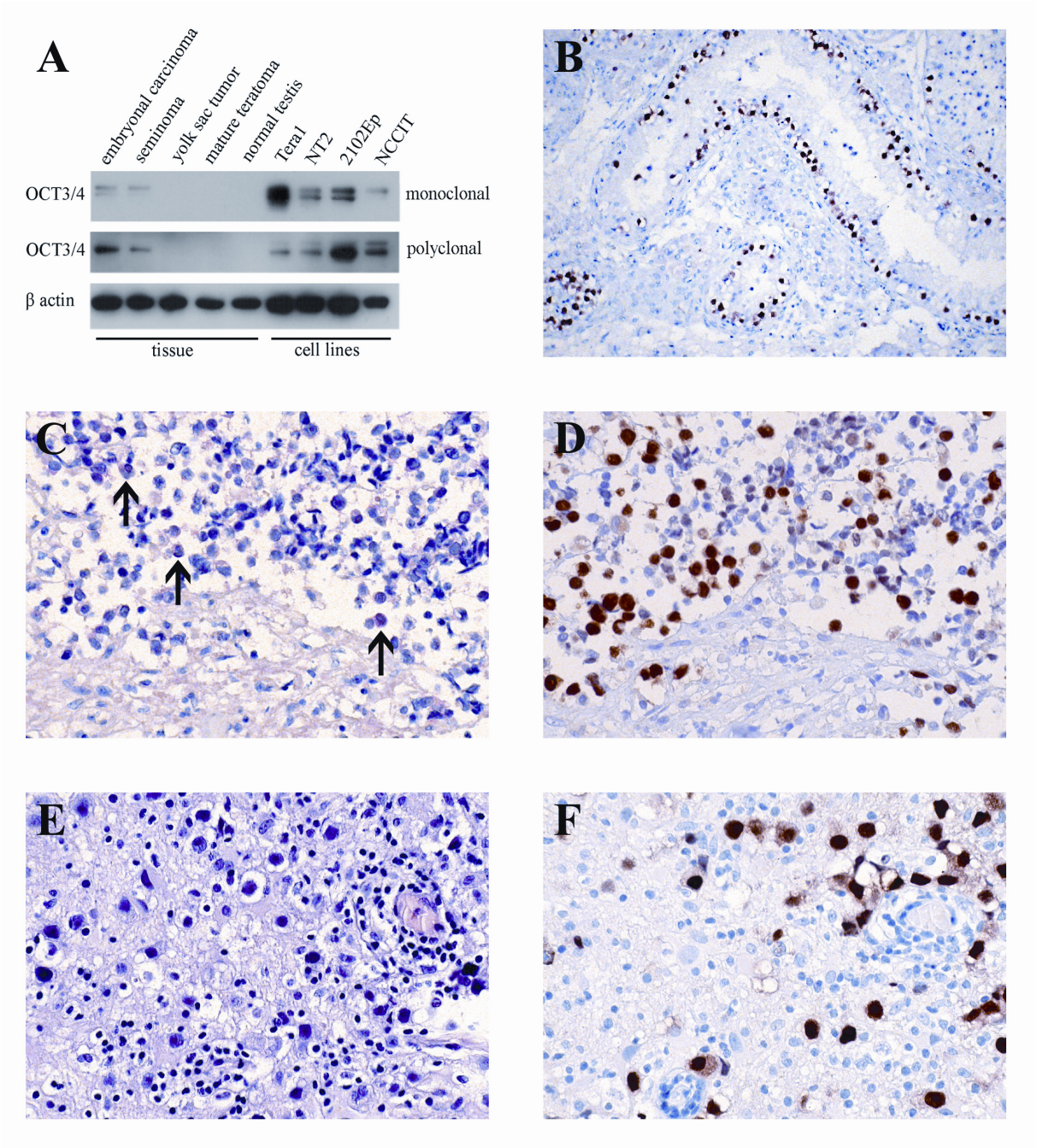

FIGURE 2. (A) Western blot analysis for OCT3/4 in different testicular germ cell tumours, derived cell lines, and normal testis. Note the specificity for seminoma and embryonal carcinoma. (B) OCT3/4 immuno-histochemistry on Bouin-fixed tissue showing positive staining of CIS. (C, D) Retroperitoneal tumour with extensive necrosis from patient 1 showing weak cytoplasmic, focally membranous staining for c-KIT in red (C, positive cells indicated with arrows) and distinct brown nuclear staining for OCT3/4 (D). (E, F) Tumour localized in the brain of patient 2, with scattered cells with clear cytoplasm and prominent nucleoli (E) with positive staining for OCT3/4 (F). *(Original position Figure 2 on page 43)*

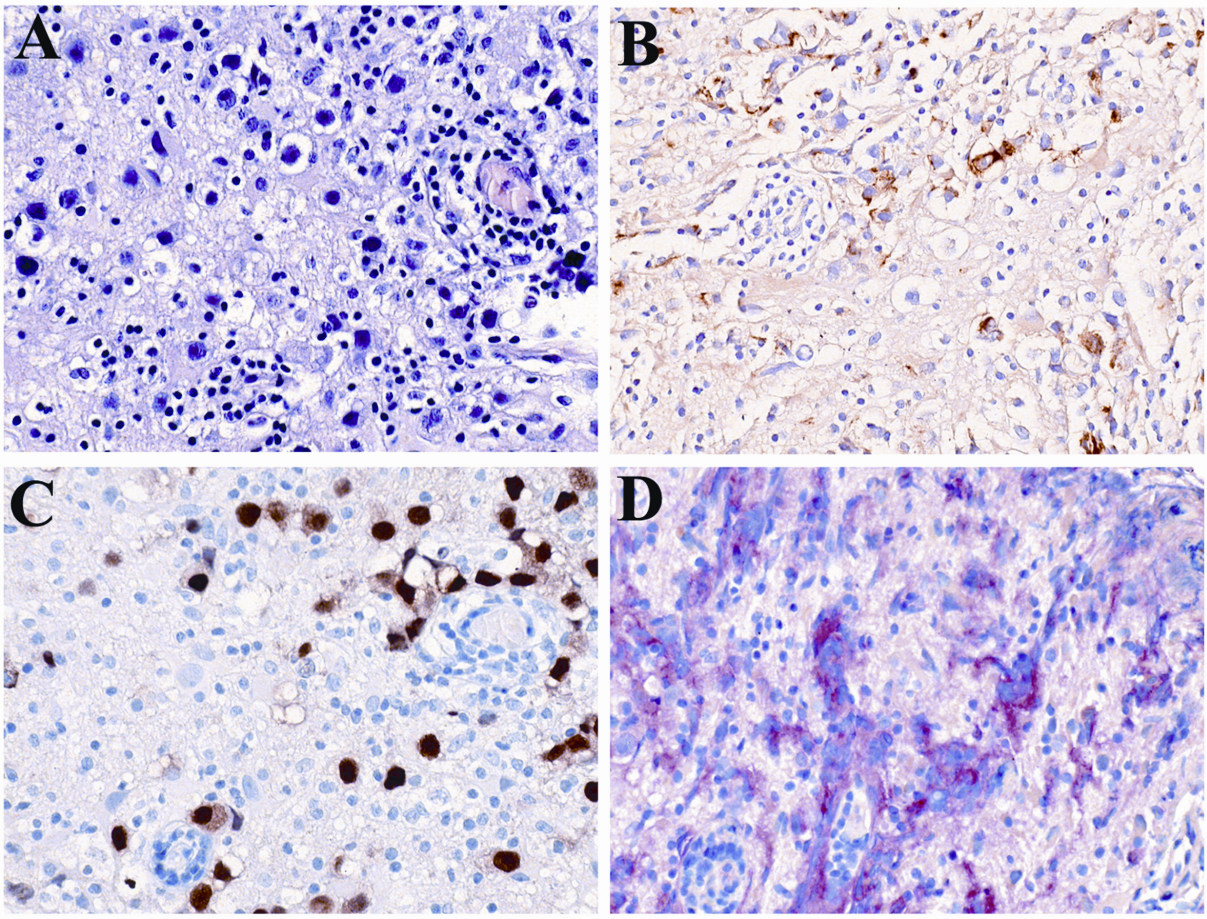

FIGURE 2. A stereotactic biopsy of the intracerebral lesion showed blast-like neoplastic cells within a mononuclear infiltrate (Figure 2A). A wide panel of markers including those for carcinoma, melanoma and primary central nervous system lymphoma was applied: CD3, CD4, CD5, ALK-1, CD19, CD20, CD79a, CD45, CD30, S-100, MELAN-A, HMB45, CD68, CD43, PLAP, hCG, AFP and CD56, all found to be negative; there was some punctuated NCL5D3 (low molecular weight keratins 8 and 18) positivity (Figure 2B). No final diagnosis could be made. Subsequently, the marker OCT3/4 became available, and 100% of tumor cell nuclei present in the biopsy clearly stained positive for OCT3/4 (Figure 2C). Recently, the stem cell factor receptor c-KIT was applied on a previously negative slide and the cytoplasm of some tumor cells was stained positive (Figure 2D). *(Original position Figure 2 on page 52)*

CHAPTER 4: OCT3/4 IDENTIFIES CIS IN TESTIS BIOPSIES

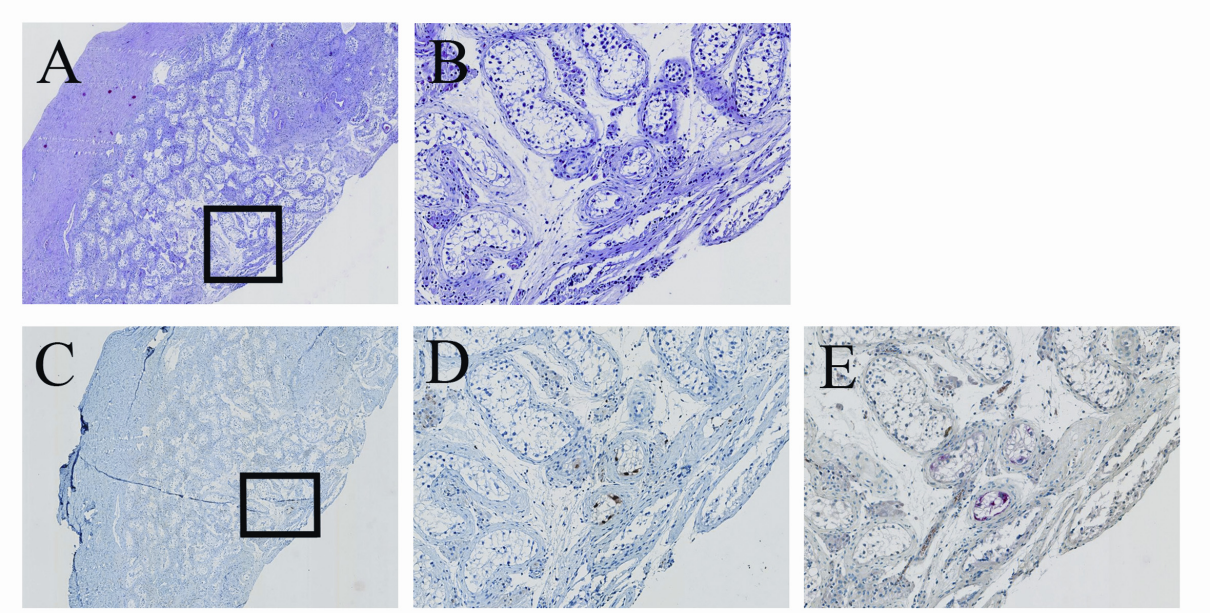

FIGURE 1. A) Histology of an open surgical biopsy of the testis stained with H&E (25x) (patient 7, Table 1). It was identified as suspected, but not proven to contain CIS; B) Higher power of suspected lesion (H & E 100x); C) Histology of the same biopsy stained immunohistochemical for OCT3/4 (25x). The region with positive cell is indicted in the square; D) Higher power of suspected lesion (OCT3/4, 100x); Only three tubules contained OCT3/4 positive cells (brown); E) PLAP immunohistochemistry of the same region, confirming the presence of CIS cells (red). *(Original position Figure 1 on page 62)*

CHAPTER 5: OCT3/4 METHYLATION IN TESTICULAR GERM CELL TUMORS

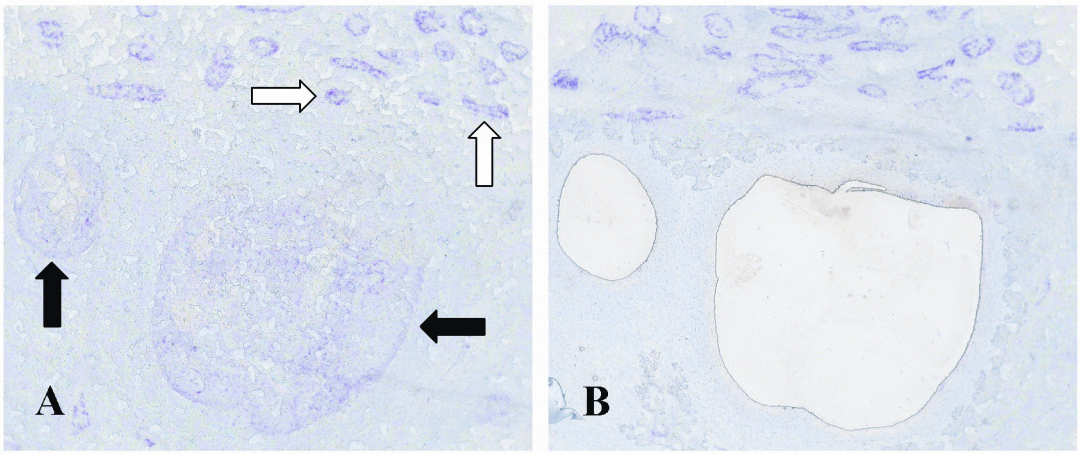

FIGURE 1. Embryonal carcinoma (black arrows) before (A) and after (B) micro-dissection. Enzymatic alkaline phosphatase stains embryonal carcinoma as well as the carcinoma *in situ* cells (white arrows). *(Original position Figure 1 on page 75)*

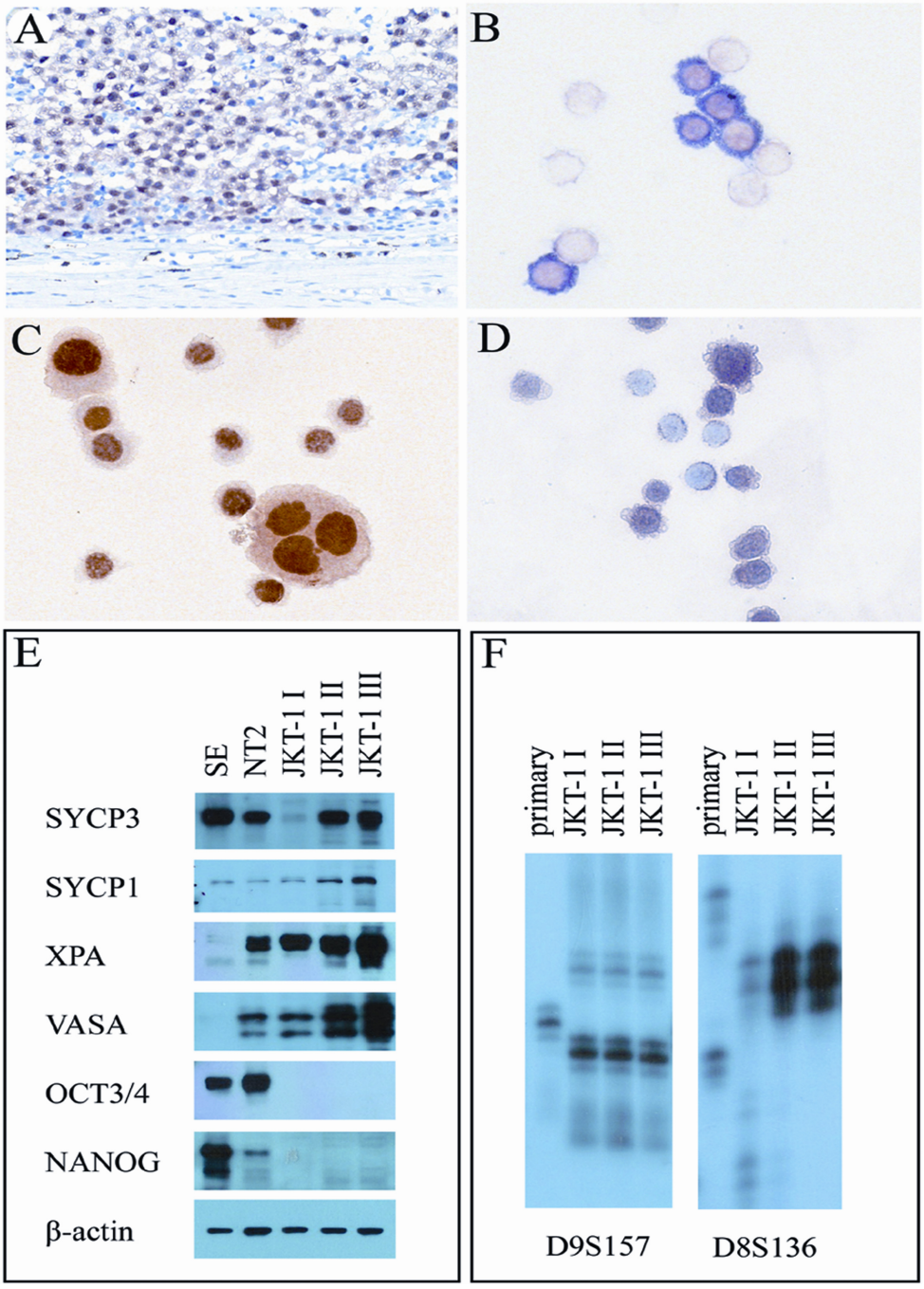

FIGURE 1. (A) Immunohistochemistry of the primary tumour for OCT3/4; immunohistochemistry of JKT-1 cells: (B) double staining for VASA (red color) and DAZL (blue color). Note the more intensive DAZL staining in the cells positive for VASA; (C) XPA; (D) SYCP3; (E) Western blot analysis of a seminoma, the EC-derived cell line NT2, and the three subclones of the JKT-1 cell lines (I, II, and III), for SYCP3, SYCP1, XPA, OCT3 / 4, NANOG and VASA, ß-actin was used as a loading control; (F) clonality analysis of the supposed primary tumour and the three JKT-1 cell lines, designated I, II and III. The following markers were applied: D9S156, D9S157, D17S786, D8S133, and D8S136. Note that the different sub-lines show the same pattern, meaning a monoclonal origin, but these were clonally unrelated to the primary tumour. The limited number of bands per marker indicate that both cell lines and primary tumour have no micro-satellite instability. *(Original position Figure 1 on page 94)*

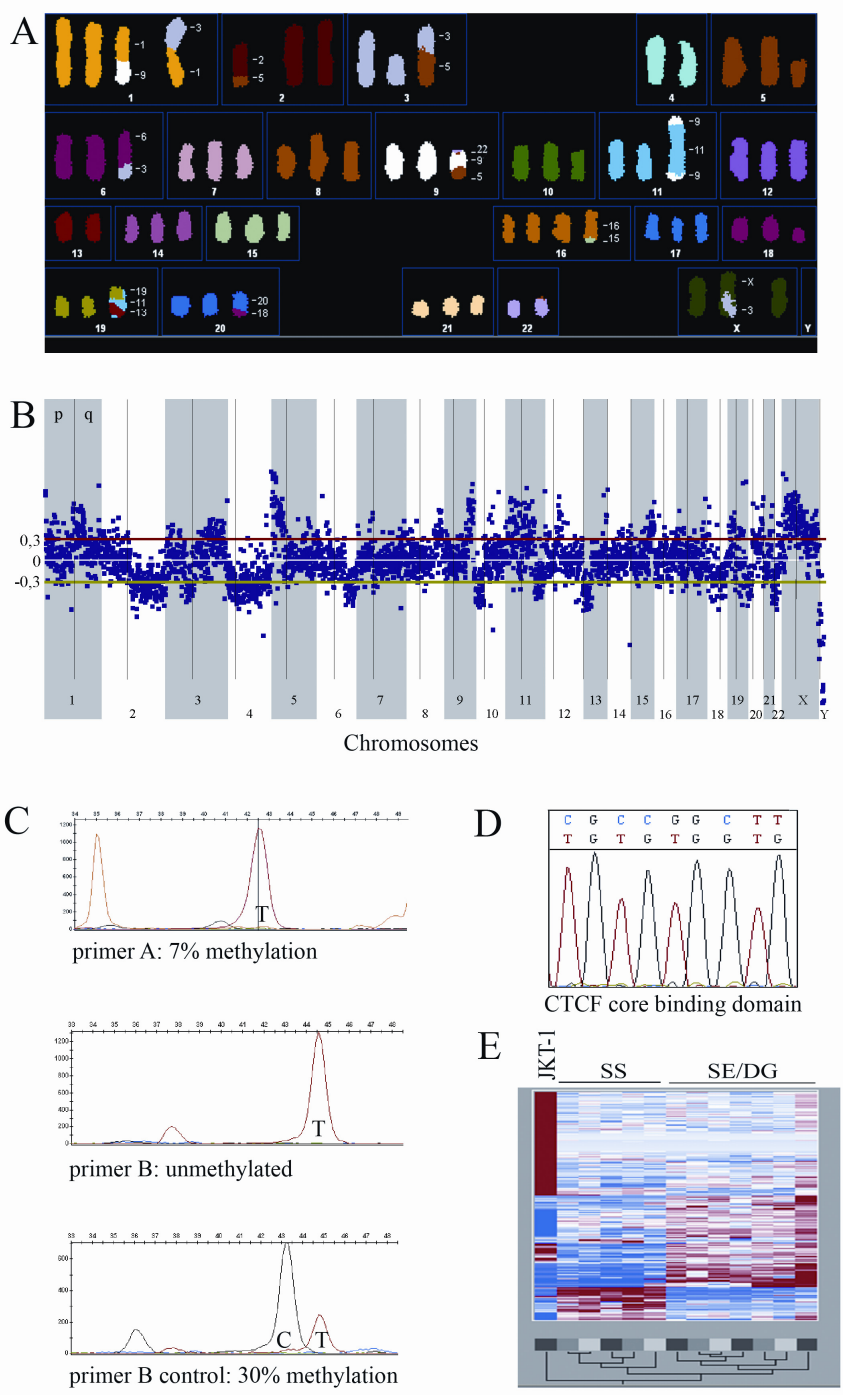

FIGURE 2 (A) Representative metaphase of spectral karyotyping analysis of JKT-1. Characteristic aberrations are loss of chromo-somes 4, 13, 18, 22 and Y; gain of X; and isochromosome 5p. The clonal chromosomal constitution based on nine metaphases is: 61-68, XX, +der(X) t(X;3)(q22;?)[7]-Y,der(1)t(1;9)(q21;q?)[8], der(1;3) (q10;p10)[8], der(2) t(2;3) (q21;q2) [2], der(2) t(2;5) (q21;?), der(3) del(3) (p?) del(3) (q?) [2], der(3;5), (p10; q10)[8],-4,+i(5)(p10), der(6) t(3;6) (?;q22)[7], del(7)(p21)[3],-9,add(9) (p11)[4], -10[3], del(10)(p1) [2], der(10) t(3;10) (?;p1?2) [6], der(11) dup(11) (p?p?) t(3;11)(?;p14) dup(11) (q?q?) t(3;11)(?;q24) [3], der(11) dup(11)(p?p?) t(9;11) (?;p14) dup(11)(q?q?) t(9;11) (?;q24) [6], -13, +der(16) t(15;16) (?;q23), -18 [7],der(18)-del(18)(p1) del(18)(q?1)[2],der(19)(19::11::13)[8], der(19)t(19;20)(?;?)[2],der(20)t(18;20) (?;?) [5], -22, +der(?)(5::9::X) [8], +der(?) (5::9::X)[2], +der(?) (?;3)[2] [cp9].
(B) Results of the 3.6 K BAC/PAC array CGH analysis of the JKT-1 cells.
(C) Methylation-sensitive single nucleotide primer extension (MS-SNuPE) of the IGF2/H19 imprinting control region of JKT-1 shows a very low methylation level by two independent primers (7% for primer A and 0% for primer B). In contrast, the sperm DNA control has a 30% methylation level; (D) Sequencing of the CTCF core binding domain on bisulfite treated DNA of JKT-1 confirms the MS-SNuPE data. All cytosines (initial sequence on top) have been converted to thymine because they were unmethylated (bisulfate treated DNA sequence below); (E). Results of Affymetrix expression profiling. Unsupervised clustering of JKT-1, 5 spermatocytic seminomas (SS), 4 seminomas (SE) and 3 dysgerminomas (DG) using Omniviz. The SE and DG could not be separated, in contrast to the SE/DG and SS. JKT-1 does not cluster with any of the tumour samples. *(Original position Figure 2 on page 96)*

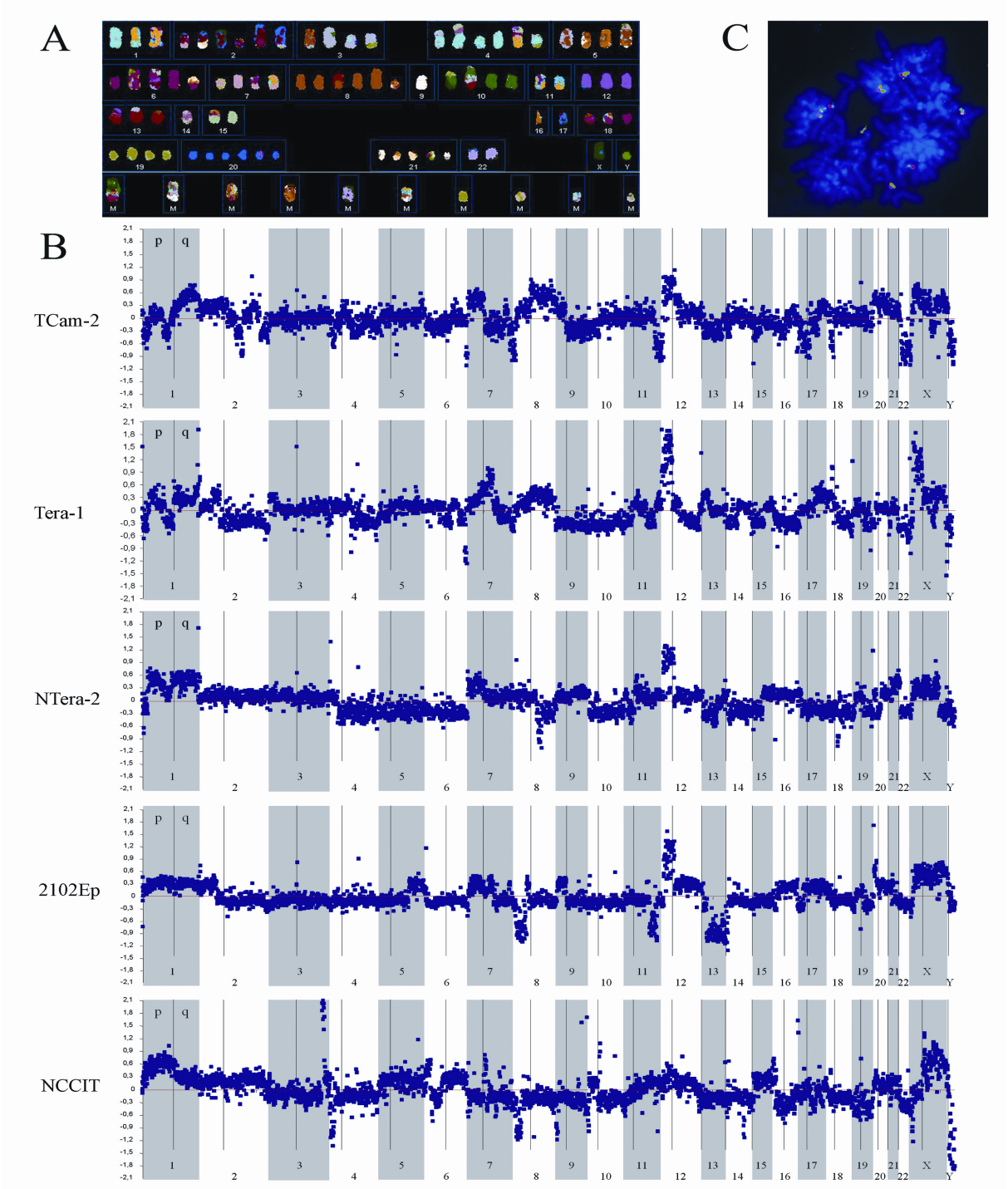

FIGURE 1. Genomic constitution of TCam-2 cells was determined by SKY analysis, array CGH and FISH. In addition, the EC-cell lines Tera-1, NTera-2, 2102Ep and NCCIT were analysed by array CGH. A) A representative metaphase of SKY analysis of TCam-2. The majority of chromosomes contains anomalies, and a large number of marker chromosomes is present; B) 3.6 K BAC/PAC array CGH analysis of TCam-2 cells and four EC-cell lines. Gain of 12p is present in all cell lines, while for example the Y chromosome is lost; C) FISH analysis confirmed the increased number of 12p copies (green signal) compared to the centromeric region of chromosome 12 (red signal) in TCam-2. *(Original position Figure 1 on page 117)*

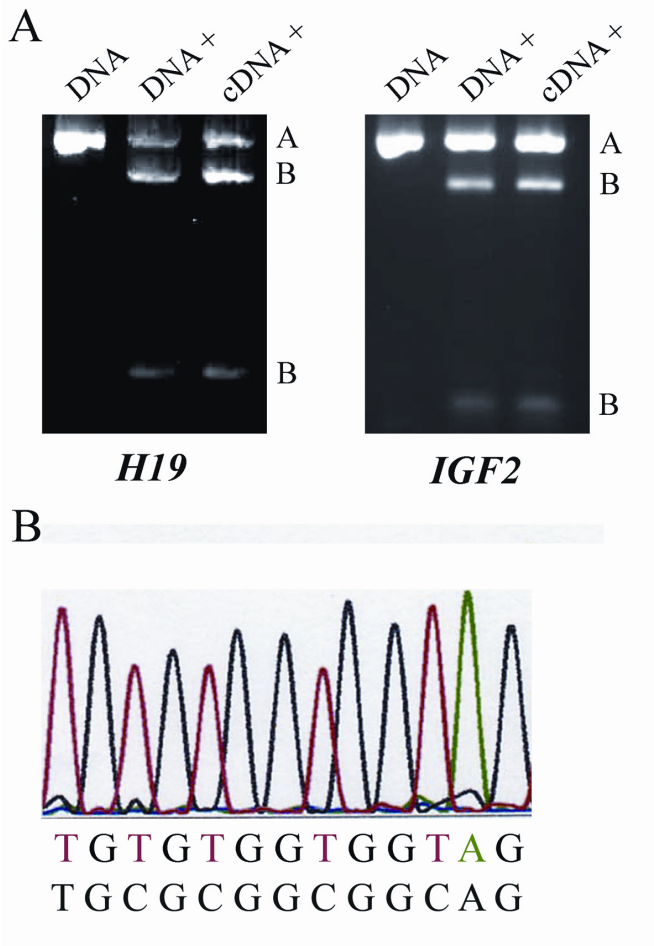

FIGURE 2. The status of genomic imprinting in the *IGF2/H19* locus was determined by polymorphic restriction enzyme digestion and the methylation status of the imprinting control region (ICR) in this locus. A) In TCam-2 both *H19* and *IGF2* genes were informative as shown by *RsaI* and *ApaI* digestion on DNA derived PCR products (DNA+). The same restriction enzyme digestions of PCR products from TCam-2 mRNA showed biallelic expression of both *H19* and *IGF2* (cDNA+), indicated by both the A and B allele; B) Bisulfite sequencing reveals that the ICR in the *IGF2/H19* locus was completely unmethylated and all cytosines were converted to thymines (indicated in red compared to the original sequence as shown below). *(Original position Figure 2 on page 118)*

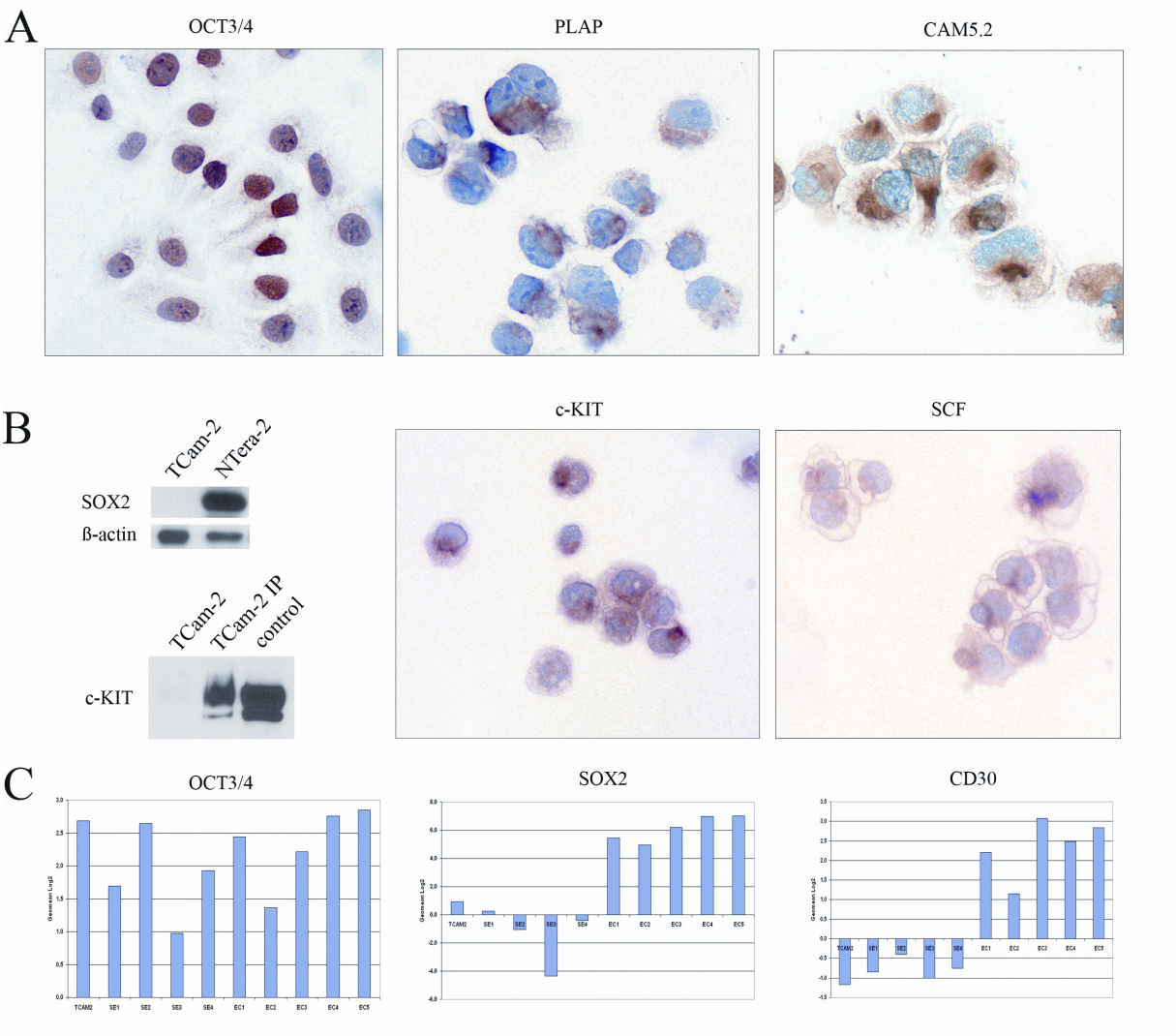

FIGURE 3. Expression of embryonic stem cell markers in TCam-2 and clinical germ cell tumor samples. A) left panel: Nuclear staining for pluripotency factor OCT3/4 in TCam-2 cells grown in a culture dish; middle panel: TCam-2 cells are positive for PLAP on cytospin preparation; right panel: dotted cytokeratin 8 (CAM5.2) staining in TCam-2 cells on cytospin preparation, as can be observed in seminoma; B) left upper panel: Western blot analysis shows expression of SOX2 in the embryonal carcinoma cell line NTera-2 and absence of SOX2 in TCam-2; left lower panel: Immunoprecipitation (IP) for c-KIT shows the expected band in TCam-2 and in F36P control total cell lysate, while c-KIT expression is very weak in total cell lysate of TCam-2; middle and right panel: immunohistochemistry for c-KIT and SCF on TCam-2 cells on cytospin preparations; C) Expression results of TCam-2 and primary human germ cell tumor samples based on genome wide (Affymetrix) expression profiling (log2 transformed data), left panel: OCT3/4 (expressed in all samples); middle panel: SOX2, and right panel: CD30, both expressed in embryonal carcinoma and low in seminoma and TCam-2. EC: embryonal carcinoma; DG: dysgerminoma; SE: seminoma. *(Original position Figure 3 on page 120)*

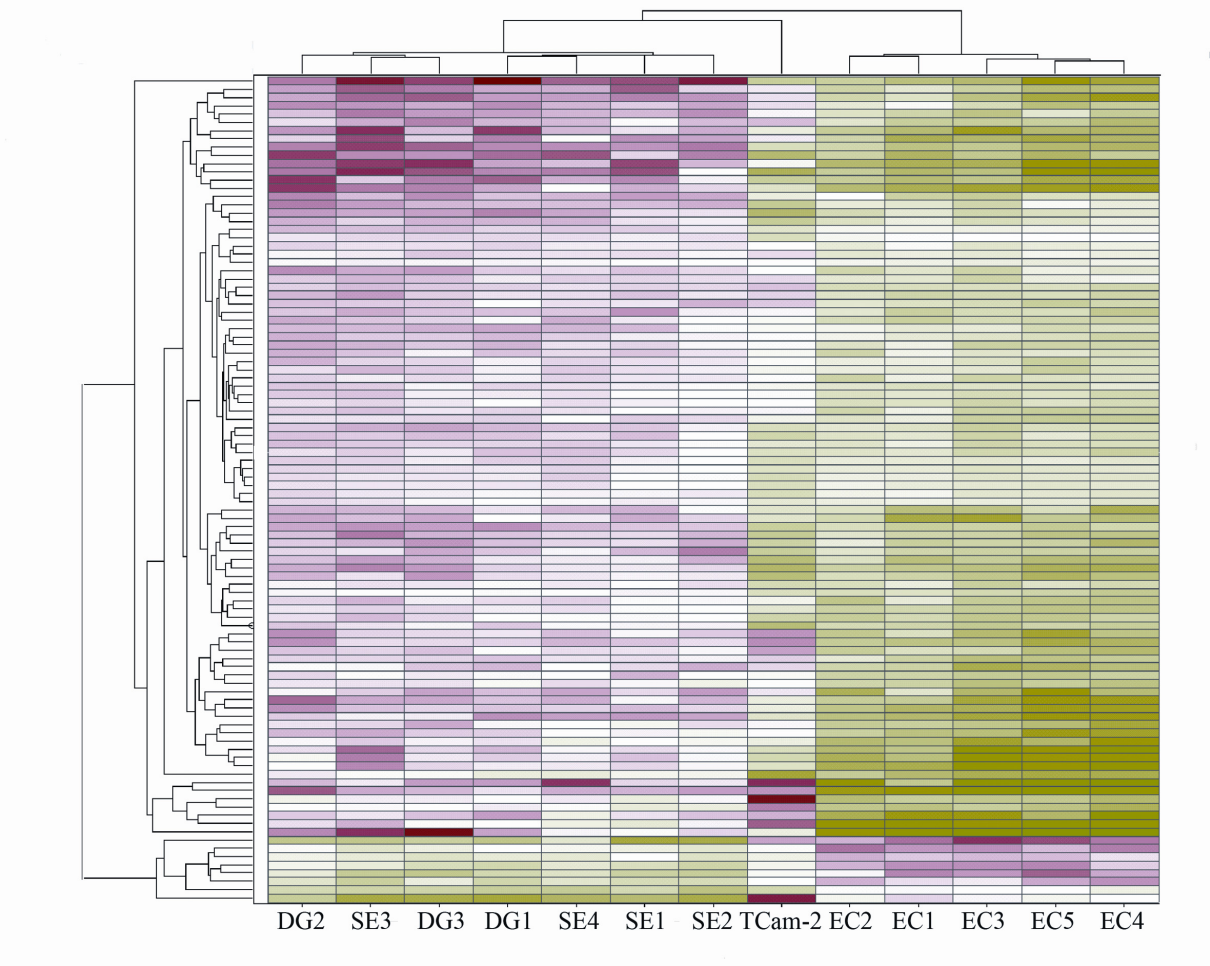

FIGURE 4. Unsupervised hierarchical clustering based on the top 100 differentiating genes between seminoma (SE)/dysgerminoma (DG) and embryonal carcinoma (EC) based on Affymetrix expression profiling to cluster the TCam-2 cell line (see supplementary data). TCam-2 clusters in the seminoma branch, separate from the EC samples. *(Original position Figure 4 on page 121)*

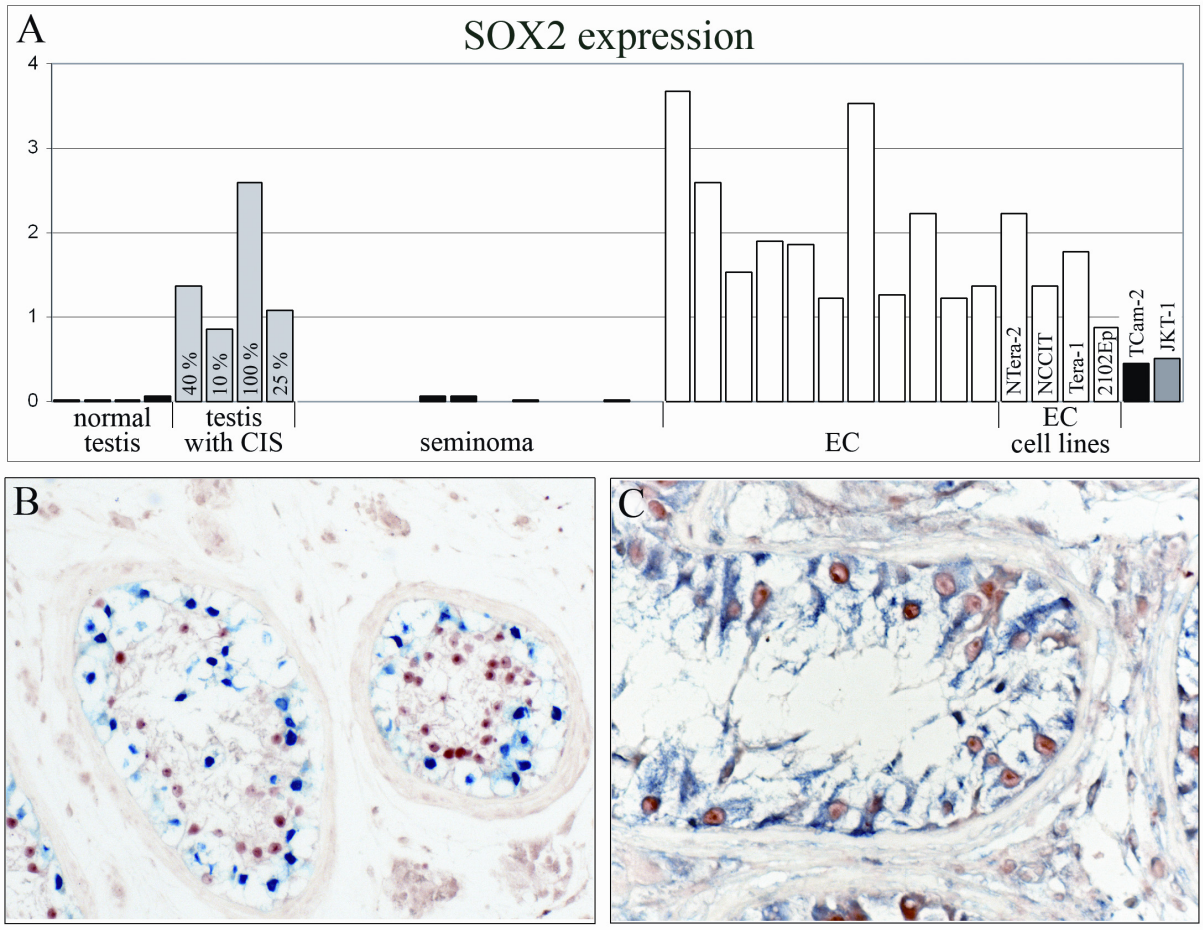

FIGURE 1. A) Quantitative RT-PCR for SOX2 on TGCT samples. Tested are the cell line JKT-1, the seminomatous cell line TCam-2 and the EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep). A high expression of SOX2 is observed in the EC samples and cell lines. In testis parenchyma containing CIS (percentage is indicated in the bars) the SOX2 mRNA expression is elevated compared to normal testis; the level of expression parallels the amount of CIS containing seminiferous tubules. TCam-2 and JKT-1 show a level of expression in between normal testis/seminoma and EC samples and cell lines. B) Double immunohistochemistry for SOX2 (red) and POU5F1 (blue) shows that SOX2 is not expressed in the POU5F1 positive CIS cells, but in a cell type more luminally located, being Sertoli cells. C) Double staining for Vimentin (blue) and SOX2 (red) combined with morphology and the position in the seminiferous tubule shows that the SOX2 positive cells are Sertoli cells. *(Original position Figure 1 on page 137)*

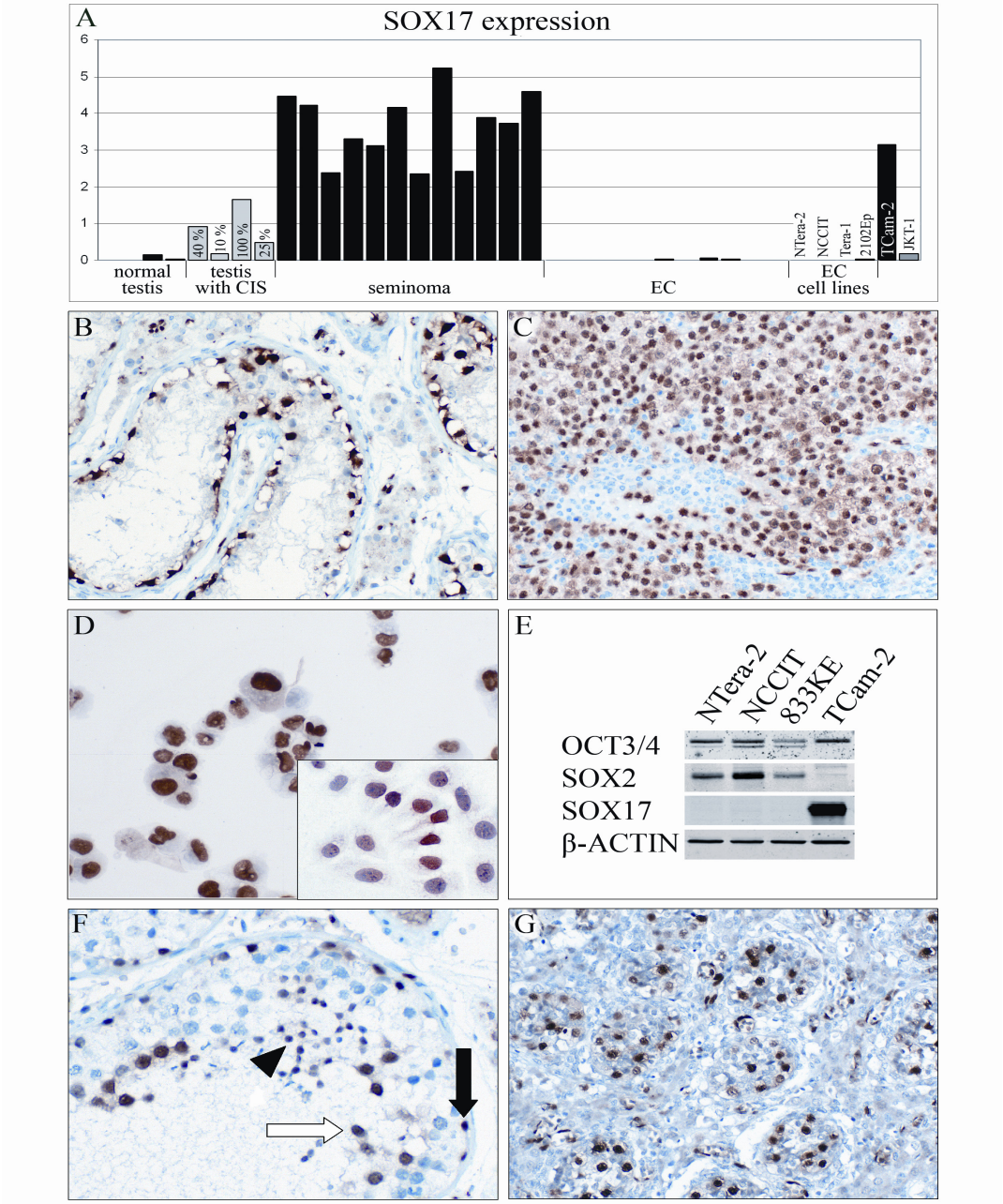

FIGURE 3. A) Quantitative RT-PCR for SOX17 on TGCT samples, cell line JKT-1, seminomatous cell line TCam-2 and EC cell lines (NTera-2, NCCIT, Tera-1, and 2102Ep) demonstrates a high expression of SOX17 in the seminoma samples and cell line TCam-2, whereas the expression is low in all other samples. Nuclear SOX17 signal (brown) in B) CIS, C) seminoma and D) the seminomatous cell line TCam-2. Inset: nuclear POU5F1 staining in TCam-2 cells. E) Western blotting shows a complementary expression pattern for SOX2 and SOX17 in TCam-2 and EC cell lines with high SOX17 expression in TCam-2 and no SOX17 expression in the EC cell lines NTera-2, NCCIT and 833KE. SOX2 is highly expressed in the EC cell lines and not in TCam-2. The embryonic stem cell marker OCT3/4 is expressed in all samples. As a loading control β-actin was used. F) SOX17 protein is present in various maturation stages of spermatogenesis: spermatogonium (black arrow), secondary spermatocyte (white arrow) and spermatid (black arrowhead), while primary spermatocytes, spermatozoa and Sertoli cells are negative. G) Nuclear SOX17 in gonocytes of an 18 week old fetus. *(Original position Figure 3 on page 139)*

ABBREVIATIONS

AFP	α-fetoprotein
CGH	comparative genomic hybridization
CHC	choriocarcinoma
CIS	carcinoma *in situ*
DNA	deoxyribonucleic acid
EC	embryonal carcinoma
ES(C)	embryonic stem (cell)
FISH	fluorescent *in situ* hybridization
GCT	germ cell tumor
hCG	human chorionic gonadotrophin
H&E	hematoxylin & eosin staining
HMG	high mobility group
ICM	inner cell mass
ICR	imprinting control region
I(T)GCNU	intratubular germ cell neoplasia unclassified
MRI	magnetic resonance imaging
miRNA	microRNA
mRNA	messengerRNA
NANOG	derived from a Celtic legend: Tir na nOg is the land of the ever young
NS	nonseminoma
OCT3/4	octamer binding transcription factor 3/4
PALGA	pathologisch anatomisch landelijk geautomatiseerd archief
PCR	polymerase chain reaction
PGC	primordial germ cell
PLAP	placental alkaline phosphatase
POU5F1	Pit-Oct-Unc family of transcription factors, class 5, factor 1
RNA(i)	ribonucleic acid (interference)
RT-PCR	reverse transcriptase polymerase chain reaction
SE	seminoma
siRNA	small interfering RNA
SKY	spectral karyotyping
SOX	SRY-related HMG box
SRY	sex determining region on the Y chromosome
SS	spermatocytic seminoma
TE	teratoma
TGCT	testicular germ cell tumor
TMA	tissue micro array
TSPY	testis-specific protein, Y-encoded
WHO	world health organization
YST	yolk sac tumor

DANKWOORD

De laatste bladzijde van mijn boekje. Geen spannende ontknoping, maar wel het stukje tekst waar iedereen benieuwd naar is. Dit boekje is het eindresultaat van het onderzoek van de afgelopen jaren waar ik met veel plezier aan heb gewerkt en waarschijnlijk ook in de toekomst mijn blik op zal richten.
Zoals een cel in het lichaam niet kan functioneren zonder de juiste stimulatie uit zijn omgeving, zo is ook dit proefschrift tot stand gekomen. Als eerste wil ik daarom mijn ouders bedanken. Pa en ma, de eigenschappen die ik van jullie geërfd heb en de steun die jullie me altijd gegeven hebben tijdens de vele omwegen die ik tijdens mijn opleiding gemaakt heb, vormen de basis van wie ik nu ben en wat ik bereikt heb. Bedankt voor alles!
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The author and the Sajama vulcano (6542 meters) in Bolivia.