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PRINCIPAL INVESTIGATOR: Yun-Fai Chris Lau, Ph.D.

CONTRACTING ORGANIZATION: Northern California Institute for Research and Education
San Francisco, California 94121

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Yun-Fai Chris Lau, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northern California Institute for Research and Education
San Francisco, California 94121

E-Mail: clau@itsa.ucsf.edu

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13. ABSTRACT (Maximum 200 Words)
The TSPY gene is the only functional gene within the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY). Expression studies demonstrated that it is aberrantly expressed in prostate cancer. This project is designed to address the role of this putative oncogene on the Y chromosome in this male-specific cancer. The objectives are: 1) to identify the oncogenic or tumor promoting domain in TSPY, and 2) to correlate TSPY over-expression with prostatic oncogenesis in transgenic mice. We have analyzed the expression of TSPY in additional cases of gonadoblastoma and samples from a tissue recombination model of human prostate cancer. Our results, so far, provide a detailed morphologic evolution of the oncogenic process in gonadoblastoma and testicular germ cell tumors and demonstrated that TSPY is co-expressed with several known germ tumor markers. We also detect TSPY expression in a hormone-induced prostate cancer model in Noble rat. Over-expression of TSPY potentiates cell proliferation. We attribute such phenomenon to a shortening of the G2/M stage of the cell cycle. We show that TSPY might exert such an effect by binding to type B cyclins. The present research provides significant insights into the probable mechanism of TSPY function in oncogenesis.

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INTRODUCTION

The TSPY gene is at tandemly repeated gene on he short arm of the human Y chromosome. Genetic mapping and complete sequencing of the human Y chromosome demonstrated that TSPY is located in the critical region harboring the only oncogenic or tumor promoting locus, termed gonadoblastoma on Y (GBY), on this male-specific chromosome. TSPY is postulated serve a function in spermatogonia by directing their division in spermatogenesis. When it is aberrantly expressed in tissues, such as the epithelial cells of the prostate, incapable of entering spermatogenesis, it exerts an oncogenic or tumor promoting effects, and in collaboration with other oncogenic events, leading to oncogenesis. The majority of our efforts for the second years are focused on the two specific aims proposed in the original application.

BODY

Task 1. To identify the oncogenic and/or tumor promoting properties of TSPY

In order to substantiate TSPY as an oncogene or tumor promoter, we have examined its expression in several new cases of gonadoblastoma and testicular germ cell tumors and examined its promoter activities in transgenic mice. The results further support this gene to be the GBY gene on the human Y chromosome.

(a) TSPY Expression in Normal Human Embryonic and Adult Testes

The human TSPY gene is expressed in human fetal, neonate and adult testes (Figure 1), primarily located in prespermatogonial cells of fetal, as early as 15 week gestation (earliest stage examined), and neonate testes, parallel to several germ cell markers, i.e. placental/germ cell alkaline phosphatase (PLAP), the stem cell factor receptor, c-Kit, and the transcriptional regulator and marker of pluripotency, Oct3/4, associated with both normal immature and malignant cells in CIS/ITGCN. In adult, TSPY is expressed most abundantly in spermatogonia and round spermatids. The embryonic expression of TSPY is significant since germ cell tumors are postulated to have their origins in primordial or early embryonic germ cells that have acquired/primed with mutational events, e.g. inappropriate expression of certain growth regulatory genes, during their differentiation, leading to development of germ cell tumors later in life. Hence, its embryonic expression in early germ cells raises the possibility that it might play a role in the early predisposition event(s) in TGCTs.

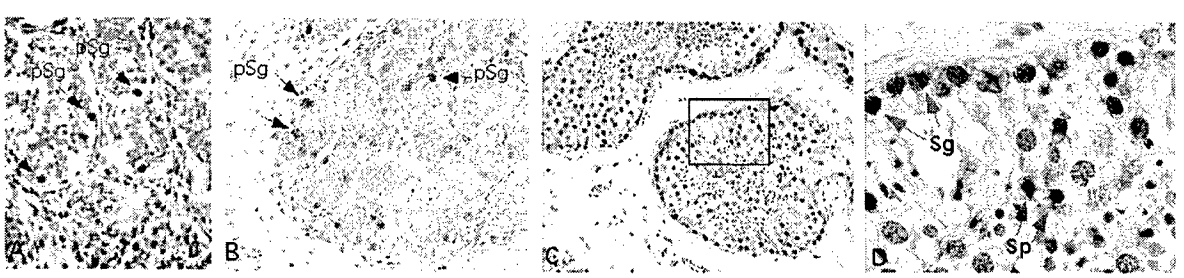

Figure 1. Immunostaining of TSPY protein in human testes using a polyclonal antibody (A-B) and a monoclonal antibody (#7, C-D). A) embryonic testis at 17-week gestation, B) 6-week neonate testis, and C-D) adult testis. TSPY is primarily located in prespermatogonial cells (pSg, A & B) of embryonic (A) and neonate (B) testes and in both spermatogonia (Sg) and round spermatids (Sp) in adult testis (D). Boxed area in C is enlarged in D.

(b) TSPY Expression in Gonadoblastoma and Testicular Seminoma

Gonadoblastoma arises from dysgenetic gonads of phenotypic females who lack a functional sex-determining gene, SRY, but acquires other Y chromosome genes incompatible with female germ cell physiology. Hence the oncogenic or cancer predisposition is caused most likely by a gain of function event. As the putative GBY gene, TSPY must be present in the XY sex-reversed patients (by deletion mapping) and be expressed in the tumorigenic site (dysgenetic gonads) to exert its mutational/or oncogenic function. To this end, we examined TSPY expression on 7 gonadoblastomas from 6 patients. The results show that TSPY is expressed in the germ cells of the tumor aggregates in all gonadoblastomas (1, and Figure 2A). The immature support cells, sex cord elements and the adjacent stromal cells are negative for TSPY. Currently, the exact etiologic mechanism for gonadoblastoma development is uncertain. The TSPY expression pattern, however, clearly demonstrates the morphological evolution of the oncogenic development (Figure 2B-G). The dysgenetic gonads of these XY sex-reversed patients harbor apparently empty follicles (Fig. 2B), probably due to failure of oogenesis. At the onset, a small number of cells at the base of the follicle might have taken on an oncogenic transformation (Fig. 2C) that expands either inward (Fig. 2D) or along the periphery of the follicle (Fig. 2E). Such oncogenic growth eventually fills up the follicle with tumor cells (Fig. 2F-G). Such confined growth might increase in size and/or merged with adjacent tumorigenic follicles (e.g. circled in Fig. 2A) or might take on a more aggressive and loose form(s) (e.g. boxed in Fig. 2A). TSPY is clearly expressed in all stages of such postulated oncogenic development, thereby positioning this putative GBY gene to be intimately involved in the process.

To determine the nature of the TSPY positive cells in the gonadoblastoma, we have performed double immunofluorescent staining with TSPY (green) and cyclin B, a potential interactive partner (Fig. 2H-J) and selected germ cell tumor markers (red), such as PLAP (Fig, 2K-M), c-kit (Fig. 2N-P) and Oct4 (Fig. 2Q-S). Our results demonstrate that cyclin B is preferentially expressed in the same tumor cells as those positive for TSPY, suggesting the possibility that both molecules might interact within these cells. Significantly, TSPY expression pattern correlates with those of the germ cell tumor markers that are inactivated and expressed at high levels in germ cell tumors, including gonadoblastoma.

To further correlate the TSPY expression at the early stage of TGCTs, i.e. CIS or ITGCN, we have examined 18 additional cases of TGCTs harboring various features, such as CIS/ITGCN, seminoma, yolk sac tumor, embryonal carcinoma. Our results show that TSPY is expressed in the tumor cells of all cases examined (e.g. Fig. 3A-E). TSPY co-localizes with other established TGCT markers, i.e. PLAP, Oct4 and c-Kit, and cell proliferative marker, Ki-67 or PCNA (Fig. 3F-M), in the same tumor cells in these TGCTs.

(c) Involvement of Tspy in an Experimental Model of Rat Prostate Cancer

To further determine if such increase in TSPY expression is also associated with prostatic oncogenesis in other experimental systems, a hormonally induced model of prostate cancer in Nobel rats (2) was analyzed similarly by immunostaining with specific antibodies. Long-term treatment of high doses of testosterone and estradiol ($T+E_2$) in this strain of rats leads to development of prostatic hyperplasia and tumors. Our results demonstrated a specific Tspy expression on morphologically normal epithelial cells in the prostate (Figure 4A-C). The signals increased gradually with increasing degrees of prostatic oncogenesis and were at the highest levels in tumor cells of high-grade cancers (Figure 4D-E). Since prostatic oncogenesis in these animals was hormonally induced, it is conceivable that Tspy expression is also regulated by androgen and/or estrogen in the rat. These observations corroborated very well with those on human prostate cancer.

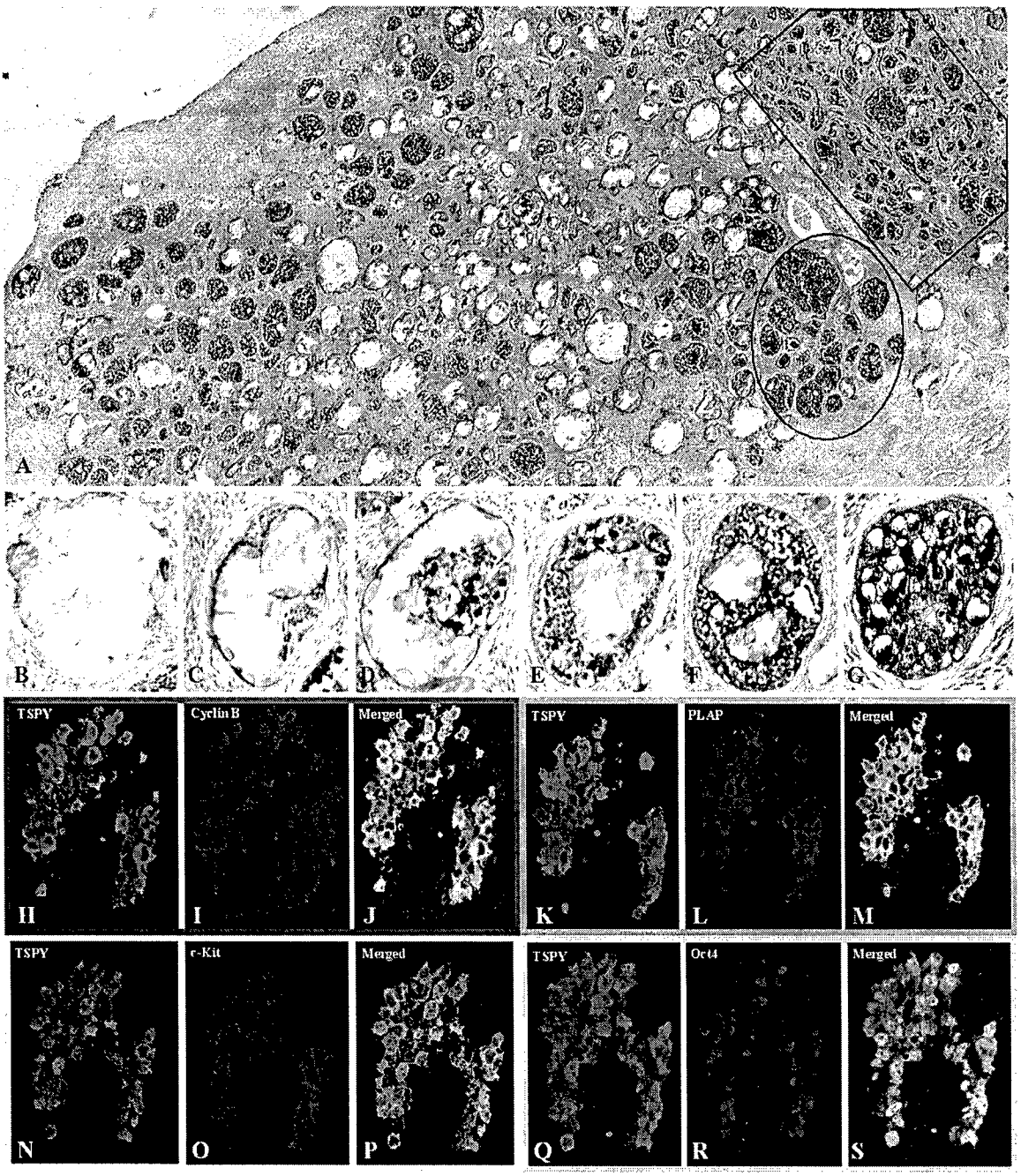

Figure 2. Co-expression of TSPY with specific germ cell tumor markers in gonadoblastoma. A. global view of a gonadoblastoma stained with TSPY antibody. B-G, Probable oncogenic sequence. Tumor cells arose at the base of an empty follicle and expanded along the periphery or towards the center, until the entire follicle is filled with tumor cells. Immunofluorescence co-localizes TSPY with various germ cell tumor markers, such as PLAP (K-M), c-Kit (N-P) and Oct4 (Q-S) on the same cells, suggesting that the tumor cells are of germ cell origin. H-J, show co-localization of TSPY with cyclin B1, a cell regulator that interacts with TSPY.

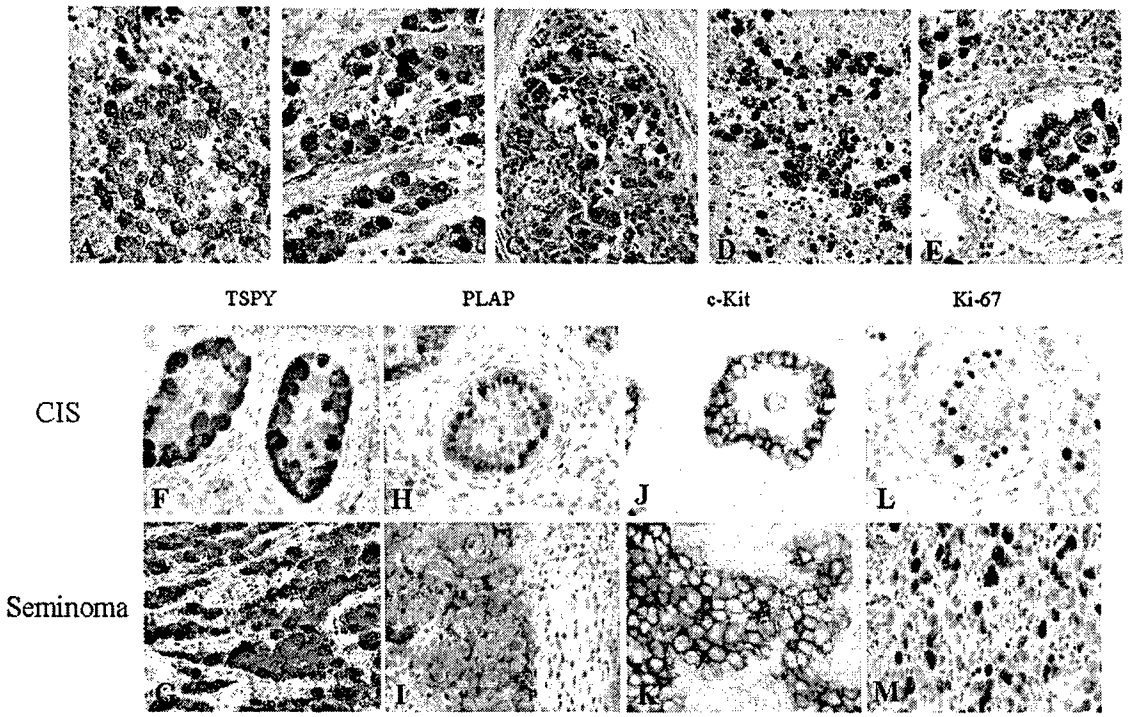

Figure 3. TSPY expression in various carinoma-in-situ (CIS) and seminomas. A-E. Random views of 5 cases of seminomas stained with TSPY antibody showing TSPY expression. TSPY proteins are localized in both cytoplasm and nuclei of these tumor cells. D is a case of lymph node metastatic seminoma. Parallel expression of TSPY (F, G), PLAP (H,I) c-Kit (J,K), and PCNA (=Ki-67, L,M) in CIS and seminoma respectively.

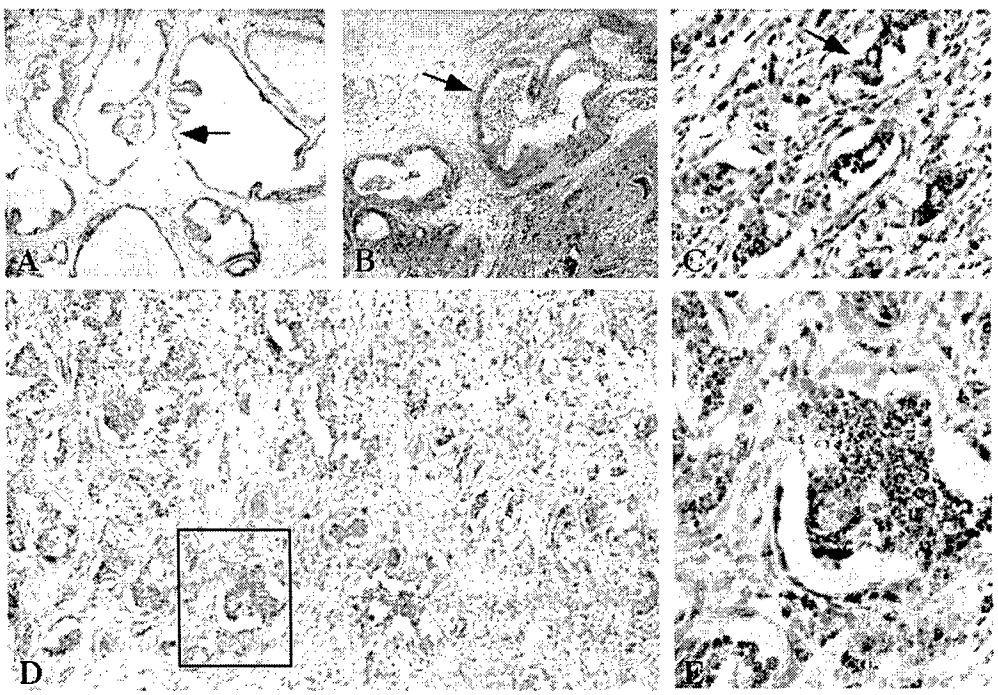

Figure 4. Expression of rat Tspy gene in hormone-induced prostate cancer in Noble rat.

(d) Structural Comparison of TSPY and its homologue, TSPY, on the X Chromosome

Four laboratories have independently isolated and characterized an X-located gene (at Xp11.22), recently designated as TSPX, that shares significant homologies to both TSPY and SET. In all instances, TSPX was isolated by exploratory cloning strategies and was designated separately as a cell division autoantigen 1 (CDA1) gene (7) and differentially expressed nucleolar TGF-b1 target (DENTT) gene (60) respectively in two laboratories that studied it in significant details. Although they were described as possessing a SET/TSPY domain in their encoded proteins, their identities as the homologue of TSPY was not obvious until the structure and mapping of these genes were studied recently (70).

TSPX was mapped to the syntenic regions harboring the homologues of other Y-genes on both human and mouse X chromosomes. Both TSPX and TSPY maintain similar gene organization (Figure 5). The TSPX gene is about 6.3-kb in size and harbors 7 exons. It encodes a ~80 kDa protein of 693 amino acids. TSPY is 2.8-kb in size and harbors 6 exons. It encodes a variety of polymorphic proteins (see Preliminary Studies) with the main product being a 35-kDa protein of 308 amino acids. The homologous regions of both TSPX and TSPY are encoded by exons 2-5 of both genes with highly conserved sequence homology and exon-intron organization. These portions of the proteins also harbor the cyclin B binding SET/NAP domain. TSPX differs from TSPY with a N-terminal proline-rich domain and a carboxyl bipartite acidic domain, encoded by exon 1 and exons 6-7 respectively. Both proteins are phosphorylated, presumably by cyclin dependent kinases and others (7). Significantly, over-expression of TSPX (CDA1) arrests cell growth. TSPX is localized to the nucleus and nucleolus of cells. Such cell growth inhibitory effects were mapped to the carboxyl acidic domain and two CDK1/2 phosphorylation sites, located at the proline-rich domain and SET/NAP domain respectively. The latter phosphorylation site is also conserved at the SET/NAP domain of the TSPY protein. Targeted mutations of both phosphorylation sites or truncation of the acidic domain of TSPX eliminates its growth inhibition. TSPX (DENTT) is expressed in a wide spectrum of tissues, with major sites in brain, lung, thymus, adrenal, pituitary, smooth muscle, testis and ovary in adult mice (66). TSPX is expressed in the heart and the primitive brain of E8 mouse embryos. The embryonic expression expands gradually to other tissues at later stages and reaches the ubiquitous pattern as in adults (69). As evidenced from results of our Preliminary Studies, described below, TSPY possesses very contrasting properties as those of TSPX and SET. Since the sex chromosomes are postulated to have evolved from a pair of identical autosomes, the properties of TSPX seem to support the notion that it has retained the housekeeping function(s) of an autosomal gene. TSPX is subjected to X inactivation in females (70), thereby preserving the dosage balance of a housekeeping gene between the sexes. However, TSPY has specialized into a role(s) in mediating male germ cell proliferation and meiosis. Hence ectopic expression or dysregulation of TSPY gene in germ cells of either a female gonad, a dysfunctional testis, or epithelial somatic cells in the prostate that are incapable of entering spermatogenesis may be responsible for predisposition to and/or initiation of oncogenesis

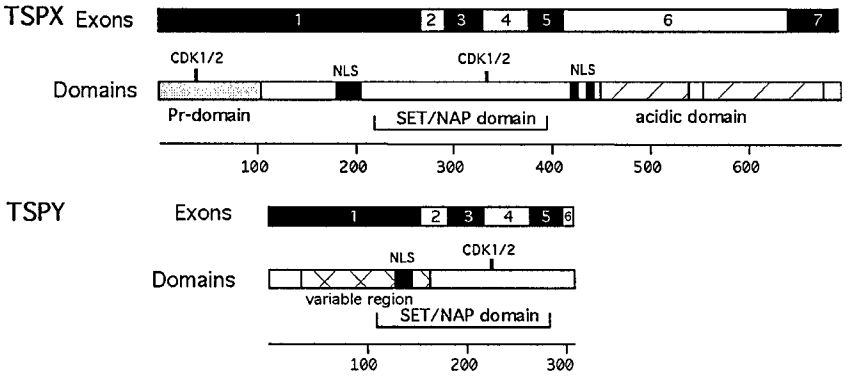

Figure 5. Exon and protein domains of TSPX and TSPY

in these tissues. This hypothesis suggests that TSPY may play the role of an oncogene or a tumor promoter in the dysgenetic gonads, testis and prostate cancers.

(e) TSPY Binds to Type B Cyclins and CDK Inhibitor $p21^{Cip1}$ and is phosphorylated by Cyclin B-CDK-1 Complex

To further understand the molecular function of TSPY, we have examined the binding between type B cyclins and TSPY protein and its isoforms deduced from alternatively spliced transcripts. GST-fusion proteins from Xenopus cyclin A, B1 and B2 (from Doug Kellogg, UCSC) were used in GST-pull down assays with in vitro synthesized and ^{35}S-labeled TSPY protein (from type 1 transcript). Results from this preliminary experiment showed that TSPY binds to both cyclin B1 and B2, but not to cyclin A1 (Figure 6A). To explore the cyclin B binding properties of TSPY, GST expression vectors were constructed with cDNA clones for human cyclin B1 (from David Morgan, UCSF) and cyclin B3 (variant 1, cloned by RT-PCR) (44). The respective GST-fusion proteins were synthesized and used in GST-pull-down assays with respective ^{35}S-lableled human TSPY proteins. Results indicated that all variant

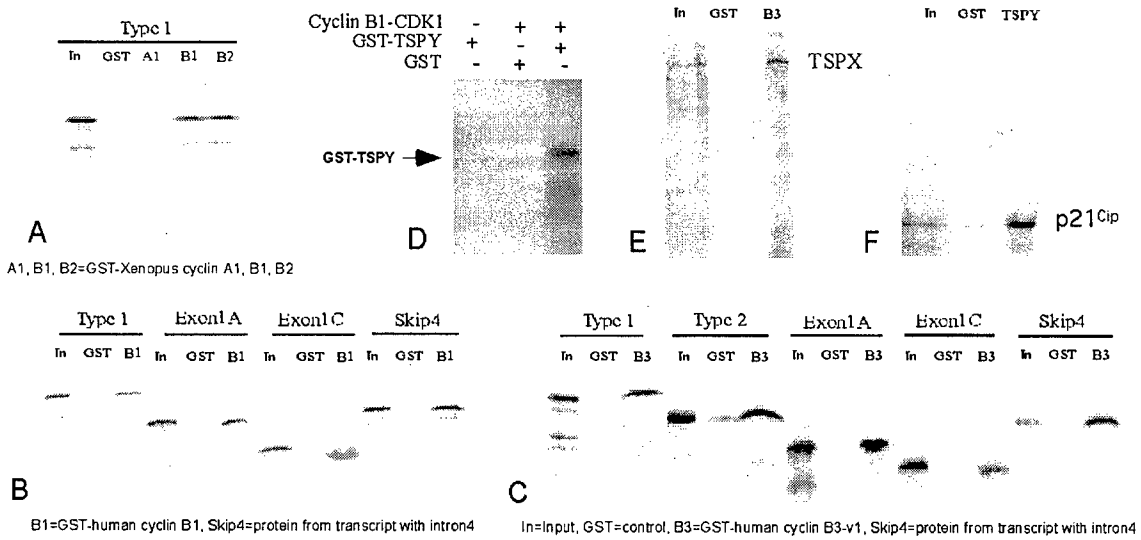

Figure 6. TSPY-cyclin B binding using GST-cyclin fusion proteins. Various TSPY isoforms (A-C) and TSPX (E) and p21Cip (F) proteins were labeled by transcription and translation reactions, bound to various GST-fusion proteins, washed eluted and analyzed by SDA-PAGE and autoradiograph. In all, these TSPY isoforms, TSPX and $p21^{Cip}$ showed preferential binding to type B cyclins. D. In vitro phosphorylatin of GST-TSPY by activated cyclin B1-CDK1 complex using ^{32}P-γ-ATP. Arrow indicates labeled TSPY.

forms of TSPY protein bound specifically with both human cyclin B1 and B3, suggesting that the cyclin B binding domain(s) were present in all the variant TSPY proteins (Figure 6B,C). Further, GST-TSPY fusion protein was phosphorylated by an activated human cyclin B1-CDK1 complex (CalBiochem Inc.) in the presence of ^{32}P-g-ATP (Figure 6D). These findings are quite interesting since cyclin B3 is an evolutionary conserved meiotic cyclin (44,57), its interaction with TSPY might suggest an important role for TSPY in regulating progression in early stages of male meiosis. Further, TSPY and cyclin B1/B2 interactions might suggest a pro-proliferative role in male germ cell lineage in both embryonic and postnatal testes. The phosphorylation of TSPY suggests that it may be one of the targets for cyclin B-CDK1 complex in vivo. Similar binding assays with labeled TSPX demonstrated that it was indeed pulled down by a GST-cyclin B3 fusion protein (Fig. 6E). Significantly, TSPX is phosphorylated at two specific sites by cyclin dependent kinase, CDK1-2 (7). The ability of TSPX to arrest cell growth could be abolished by mutating these two sites. Based on our structural analysis of TSPX and TSPY (Fig. 5), one of the phosphorylation sites at the SET/NAP domain is conserved in TSPY proteins. Since over-

expression of TSPY potentiates cell growth and proliferation, our results suggest that these homologues on the sex chromosomes might serve opposite functions in cell cycle regulation and are mediated by cyclin dependent kinases.

Previously, SET had been demonstrated to interact with the inhibitor $p21^{Cip1}$ for CDK and modulates the cell cycle progression (6,16). To explore the possibility of a similar interaction between $p21^{Cip1}$ and TSPY, similar pull-down assay with a GST-TSPY and ^{35}S-lableled $p21^{Cip1}$protein showed that $p21^{Cip1}$ indeed bound to and was pulled down by the TSPY fusion protein (Fig. 6F).

Task 2. To Correlate TSPY Over-Expression with Prostatic Oncogenesis in Transgenic Mice

To examine the effects of aberrant and over-expression of TSPY in prostatic tissues, we have adopted the Cre-LoxP transgenic activation system to manipulate the expression of a human TSPY transgene. In this scheme, there are two transgenic components, the transactivator and responder. The transactivator is a transgenic mouse line harboring a prostate-specific Cre recombinase transgene and the responder mice harbor a transgene with inhibitory sequences, flanked by two LoxP sites, followed by a TSPY-ERES-EGFP expression cassette. The subjects are double transgenic male mice, in which the Cre gene will be expressed specifically in their prostates, which, in turn, cleaves the inhibitory sequences flanked by the two LoxP sites, thereby positioning the TSPY-IRES-EGFP expression cassette directly under the control of an ubiquitous and strong actin promoter. This recombination will therefore express TSPY and EGFP in high levels in the prostate.

(a) Establishment of the Cre-LoxP Transgene Activation System

To establish the Cre-LoxP transgene activation system, we have generated two lines of transgenic mice harboring a Cre recombinase transgene directed by a 2.4-kb TSPY promoter. Animals from these transgenic lines are crossed with a reporter line, Z/EG that harbors a lacZ gene flanked by two LoxP sites, followed by the EGFP expression cassette. According to this scheme, TSPY directs the expression of a Cre recombinase that cleaves the loxP sites flanking an inhibitory sequence, thereby activating an EGFP reporter gene in the double transgenic mice. So, expression of EGFP is an indicator for TSPY promoter activities.

Using this strategy, we demonstrate that TSPY promoter is indeed active in germ cell lineages of both sexes in embryonic, neonate (at low levels) and adult (at high levels) animals, similar to the TSPY expression patterns in humans. In particular, EGFP is clearly located in the late primary and secondary follicles of female ovary (Fig. 7), resembling the immunostaining results with gonadoblastoma samples (Fig. 7). Results from these studies firmly establish TSPY as the gene for GBY on the Y chromosome.

(b) Establishment of Prostate-Specific Activation of TSPY Gene

To achieve this goal, we have obtained the transactivator line, PB-Cre4, from Dr. Pradip Roy-Burman, University of California, Los Angeles. We have also generated 9 transgenic founders harboring the floxed TSPY-IRES-EGFP transgene. Six transgenic lines are obtained with these founders, but only one line (#5) expresses the β-galactosidase, indicating functionality of the expression cassette. In order to assemble the system, we have crossed this responder line with the PB-Cre4 transactivator line. Today, we have generated at least 20 double transgenic male transgenic mice, but none of them seem to express the TSPY or EGFP proteins at high enough levels for immunostaining detection. This is in contrast to the results of our TSPY-Cre and Z/EG

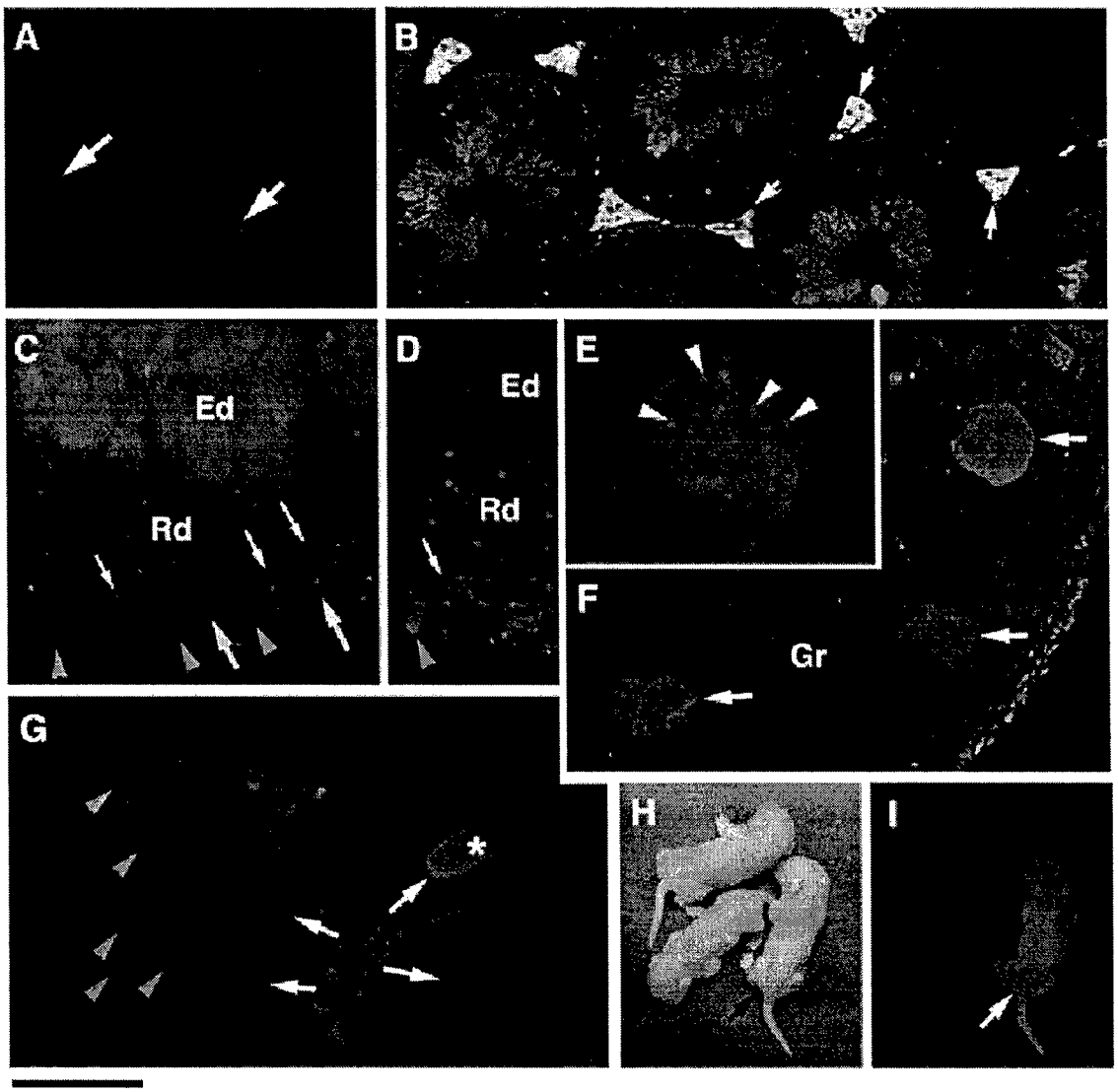

Figure 7. TSPY promoter directed expression of EGFP reporter in adult testis (A-C) and ovary (E-G) using a Cre-LoxP gene activation system. EGFP can be observed directly in dissected testis (A) and ovary (E). EGFP is primarily expressed in spermatids (B-C) and ovarian follicles (F-G). D is a negative control. The EGFP-labeled sperms and oocytes can participate in a fertilization process and transmit the transgene to offspring (H-I).

combination that works very well with this transgenic activation system. To evaluate the probable reason for this problem, we have crossed the respective lines with some reference lines, i.e. PB-Cre4 with the Z/EG responder line and TSPY-Cre with the floxed TSPY-IRES-EGFP line. These crossing and breeding schemes are time-consuming and take considerable amount of time to obtain results for further evaluation. The results suggested that the PB-Cre4 transgene expression is low for an efficient recombination, while the floxed TSPY-IRES-EGFP responder line seems to be inefficient for activation of TSPY and EGFP proteins.

FUTURE DIRECTIONS

For the next funding period, we plan to focus on the transgenic mouse studies and explore alternative strategies to over-expression TSPY in the prostates of its hosts. One alternative strategy will be to use prostate-specific promoters of different strengths, i.e. a short and a enhanced rat probasin promoter, that have been successfully employed to express the c-Myc oncogene at low and high levels respectively in the prostates of transgenic mice. Due to time limitation, this alternative strategy should be most appropriate for the remainder of the project. We will vigorously explore this approach in the coming year. In the meantime, we plan to complete the cell proliferation assays by analyzing the effects of over-expressing various TSPY proteins with specific mutations at specific domains in culture cells.

KEY RESEARCH ACCOMPLISHMENTS

- Establish TSPY to be the putative gonadoblastoma gene.
- Demonstrate Tspy gene expression in an experimental model of prostate cancer in Nobel rat
- Show the human TSPY promoter being capable of directing the expression of a transgene in germ cell lineages of both sexes.
- Evaluate the structural similarities and differences between TSPY and an X-homologue, TSPX.
- Identify the key TSPY domains involved in interaction with type B cyclins.

REPORTING COTCOMES

1. Honecker F, Stoop H, de Krijger R, Lau Y-FC, Castrillon D, Oosterhuis JW, Bokemeyer C and Looijenga LHJ (2004). Pathological implications of the expression of markers of testicular carcinoma in situ by fetal germ cells. J Pathol, 203:849-857.

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3. Kido T and Lau Y-FC (2005). The promoter of the putative GBY gene on the Y chromosome is capable of directing a transgene expression in female mouse germ cells. Genesis, submitted.

CONCLUSION

We have now established strong evidence supporting TSPY to be the gene for GBY, the only oncogenic locus on the human Y chromosome. Its expression in prostate cancer suggests that it might play a significant role in the multistep oncogenic process in prostate cancer. We have now initiated and performed considerable amount of research in establishing the Cre-LoxP transgene activation system to manipulate the expression of a TSPY transgene in the prostate of the host animals. These efforts result in the demonstrating the promoter of the human TSPY gene being capable of directing transgene expression in germ cell lineages of both sexes. The ovarian germ cell expression of the transgene suggests that the TSPY promoter, and hence the TSPY gene, could be expressed before gonadoblastoma actually develops. This finding supports the notion that TSPY

has a primary role in gonadoblastoma. These results certify TSPY as a putative oncogene in this man-only chromosome.

SO WHAT

GBY is the only oncogenic locus on the Y chromosome. Equating TSPY to be the gene for GBY is significant in establishing a role for this chromosome in male-specific cancers, such as TGCTs and prostate cancer. Successful implementation of the transgenic mouse studies in this project will be critical in establishing the TSPY gene in prostate cancer.

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Cytogenetic and Genome Research

Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer

Y.-F.C. Lau, H.W. Lau and L.G. Kömüves

Department of Medicine, VA Medical Center, University of California, San Francisco, San Francisco CA (USA)

Abstract. The contribution of specific genes on the Y chromosome in the etiology of prostate cancer has been undefined. Genetic mapping studies have identified a gonadoblastoma locus on the human Y chromosome (GBY) that predisposes the dysgenetic gonads of XY sex-reversed patients to tumorigenesis. Recently a candidate gene, the testis-specific protein Y-encoded (TSPY) that resides on the GBY critical region, has been demonstrated to express preferentially in tumor cells in gonadoblastoma and testicular germ cell tumors. TSPY shares high homology to a family of cyclin B binding proteins and has been considered to possibly play a role in cell cycle regulation or cell division. To address the possible involvement of the TSPY gene in prostate cancer, both in situ mRNA hybridization and immunohistochemistry techniques were used to study the expression of this putative GBY gene in prostate specimens. Our results demonstrated that TSPY was expressed at low levels in normal epithelial cells and benign prostatic hyperplasia (BPH), but at elevated levels in tumor cells of prostate cancers at various degrees of malignancy. Sequence analysis of RT-PCR products obtained from both prostatic and testicular tissues using specific primers flanking the open reading frame of the TSPY mRNA revealed a complex pattern of RNA processing of the TSPY transcripts involving cryptic intron splicing and/or intron skipping. The variant transcripts encode a variety of polymorphic isoforms or shortened versions of the TSPY protein, some of which might possess different biochemical and/or functional properties. The abbreviated transcripts were more abundant in prostatic cancer tissues than the testicular ones. Although the exact nature of such variant TSPY transcripts and proteins is still unclear, their differential expression suggests that the TSPY gene may also be involved in the multi-step prostatic oncogenesis besides its putative role in gonadoblastoma and testicular seminoma.

Failure in testis determination in the presence of the whole or a portion of the Y chromosome predisposes XY sex-reversed individuals to gonadoblastoma development early in life (Scully, 1953, 1970). In 1987, David Page proposed that there is a locus on the Y chromosome, termed gonadoblastoma on the Y chromosome or GBY, that harbors a gene(s) that promotes tumor formation in the dysgenetic gonads of XY sex-reversed females and females with 45,X/46,XY mosaic karyotype (Page, 1987; Lau, 1999). Deletion mapping had localized this locus to small (~1–2 Mb) regions on the short and long arms proximal to the centromere of the Y chromosome. Among the genes located on this critical region for GBY, the Y-specific repeated gene, the testis-specific protein Y-encoded (TSPY) (Zhang et al., 1992; Schnieders et al., 1996) is one of the most significant candidates for this oncogenic or tumor-promoting locus. First, most of the functional copies of the TSPY gene are located in the GBY critical region (Salo et al., 1995; Tsuchiya et al., 1995; Vogt et al., 1997; Stuppia et al., 2000; Rottger et al., 2002; Skaletsky et al., 2003). Second, expression analyses clearly demonstrated high levels of TSPY expression in gonadoblastoma tissues (Hindenbrand et al., 1999; Lau et al., 2000). Additional experiments also detected similarly high levels of TSPY expres-

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Request reprints from Dr. Chris Lau
Laboratory of Cell and Developmental Genetics
Department of Medicine, VA Medical Center, 111C5
University of California, San Francisco
4150 Clement Street, San Francisco, CA 94121 (USA)
telephone: 415-379-5526; fax: 415-750-6633; e-mail: clau@itsa.ucsf.edu

Present address of L.G.K.: COR Therapeutics, Inc.
256 East Grand Avenue, South San Francisco, CA 94080 (USA).

KARGER Fax + 41 61 306 12 34
E-mail karger@karger.ch
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sion in the tumor cells of testicular seminoma and carcinoma-in-situ (Schnieders et al., 1996; Lau et al., 2000). Third, TSPY encodes a putative cyclin B binding protein that may be involved in regulation of cell proliferation. These data, taken together, suggest that TSPY may play an oncogenic or cancer promoting role in gonadoblastoma and testicular cancer.

To evaluate the role of the male-specific Y chromosome in prostatic oncogenesis, we had independently examined the expression of 31 Y chromosome genes on a panel of prostate samples diagnosed with benign prostatic hyperplasia (BPH), low and/or high grade carcinoma, and established prostatic cell lines (Lau and Zhang, 2000). This study identified several Y chromosome genes that were heterogeneously and/or differentially expressed among the prostatic samples and/or cell lines. In particular, TSPY was preferentially expressed in prostatic cancer samples. Its expression in the prostatic cancer cell line, LNCaP, was responsive to androgen induction, suggesting that it may be involved in this hormonally sensitive cancer. These findings are significant because as a candidate for GBY, TSPY could potentially play additional roles in the etiologies of other male-specific cancers, such as testis and prostate cancers. To explore this possibility, we have performed detailed expression studies on various prostate tumor samples using both in situ mRNA hybridization and immunohistochemistry techniques. Our data show a basal level of TSPY expression in normal epithelia, and elevated levels in cancers of increasing Gleason grades. RT-PCR and sequence analyses of TSPY mRNAs from both prostatic and testicular tissues revealed a variety of alternatively spliced variant transcripts whose ORFs would encode either slightly polymorphic isoforms or shortened versions of the TSPY protein. Although the exact nature of these polymorphic TSPY proteins is still unknown, their identification raises the possibility that one or a few of the TSPY isoform(s) may play a role in the development and/or progression of this male-specific cancer.

Table 1. Prostate and testis samples

A. Prostate samples for in situ hybridization and immunohistochemistry

Patient/Sample Number	Age	Gleason Grade[a]	Source
S2312	62	CaP, 3 + 3 = 6	VAMC-SF
S3104	69	CaP, 4 + 5 = 9	VAMC-SF
S2249	64	CaP, 3 + 3 = 6	VAMC-SF
S1748	54	BPH	VAMC-SF
S2011	78	BPH	VAMC-SF
S1050	41	CaP, 5 + 5 = 10	VAMC-SF
S2337	61	CaP, 4 + 5 = 9	VAMC-SF
S482	82	CaP, 5 + 4 = 9	VAMC-SF
S2699	68	CaP, 2 + 3 = 5	VAMC-SF
S1868	58	CaP, 3 + 2 = 5	VAMC-SF

[a] CaP, Prostatic adenocarcinoma.

B. Testis and prostate samples for RT-PCR analysis, cloning and sequencing

Patient/sample number (abbreviated name)	Age	Clinical conditions and/or Gleason grade[a]	Source[b]
16762A1-E (P762CaP)[c]	60	CaP, 3 + 4 = 7	CHTN
16762A2-A (P762NL)[c]	60	BPH of 16762A1-E	CHTN
00-05-A143B (P143B)[c]	80	CaP, 3 + 3 = 6	CHTN
00-07-A218A (P218A)[c]	67	CaP, 3 + 4 = 7	CHTN
00-08-A298B (P298B)	68	CaP, 3 + 4 = 7	CHTN
20475A1A (P475)[c]	64	CaP, 3 + 3 = 6	CHTN
P1 (P1)[c]	72	BPH	CHTN
4001179J (P179)	72	BPH	CHTN
90-02-D020 (T20)	60	normal testis	CHTN
89-09-091 (T91)[c]	65	normal testis	CHTN
89-05-036 (T36)[c]	68	normal testis	CHTN
88-02-045 (T45)[c]	74	testicular mass	CHTN
95-10-H003 (T3)[c]	40	testicular seminoma	CHTN
T1 (T1)[c]	25	normal testis	UCSF
T4 (T4)[c]	36	testicular seminoma (non-tumor portion)	UCSF

[a] CaP, Prostatic adenocarcinoma; BPH, benign prostatic hyperplasia.
[b] CHTN, Co-operative Human Tissue Network; UCSF, Tissue Core, University of California, San Francisco.
[c] cDNA clones were sequenced in this project.

Materials and methods

In situ mRNA hybridization and immunohistochemistry

Five- to seven-micrometer sections were prepared from ten paraformaldehyde-fixed and paraffin-embedded pathological specimens from the archives of the Anatomic Pathology Section, VA Medical Center, San Francisco. The samples were derived from prostatectomy and needle biopsy procedures and were diagnosed to harbor foci of benign prostatic hyperplasia (two cases) and adenocarcinoma (nine cases with Gleason grades ranging from 2+3 to 5+5) by the attending staff pathologists at the VA Medical Center, San Francisco (Table 1A). This and subsequent studies were conducted according to protocols approved by the Institutional Committee on Human Research.

In situ mRNA hybridization was performed with the above prostate tissue sections as previously described (Stelnicki et al., 1998). Briefly, the cDNA of the human TSPY gene was subcloned in the pAR3038 plasmid in either transcription orientation of a T7 promoter. The respective recombinant plasmids were digested with *Bbs*I that truncated the cDNA at the end distal to the T7 promoter. Anti-sense and sense RNAs were synthesized with a riboprobe kit (Roche Biochemicals) using the T7 bacteriophage RNA polymerase in the presence of biotin-16-UTP. The riboprobes were concentrated by ethanol precipitation and were used at 20 µg/ml in the hybridization procedure. The sections were pre-treated with HCl, proteinase K, and acetic anhydride. They were prehybridized and hybridized with the respective biotin-labeled probes in a 50% formamide buffer at 37 °C overnight. The sections were then washed and treated with RNase to eliminate unhybridized single-stranded RNA probes. A tyramide amplification step was used to enhance the hybridization signals (Speel et al., 1999) before the ABC-peroxidase-DAB substrate reactions (Vector Laboratories Inc.). The slides were counter-stained with methyl green and examined under a Zeiss Axiophot photomicroscope.

Immunohistochemistry was performed as previously described using a specific antibody against the human TSPY protein (Lau et al., 2000). The binding of the primary antibody was detected with an affinity-purified goat anti-rabbit IgG and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents and substrate kits (Vector Laboratories Inc., Burlingame, CA). For the brown and red signals, the DAB and VECTOR RED kits were used with the ABC-peroxidase and ABC-alkaline phosphatase reagents respectively. An antibody against the human cyclin B1 was a gift from Drs. Catherine Takizawa and David Morgan, UCSF. The monoclonal antibody, PC10, against the proliferating cell nuclear antigen (PCNA) was purchased from Dako Corporation and used according to the procedure provided by the vendor. All three antibodies, TSPY, cyclin B1 and PCNA, had previously been demonstrated to be specific to their respective antigens in immunohistochemistry (Lau et al., 2000). The slides were counter-stained briefly with hematoxylin-eosin and examined with a Zeiss Axiophot photomicroscope as above.

RT-PCR cloning, DNA sequencing and anlaysis

Frozen prostatic and testicular normal and cancer tissues were obtained from either the Cooperative Human Tissue Network or the Tissue Core Laboratory of the Cancer Center at the University of California, San Francisco (Table 1B). Their classifications were based on pathological examination of parallel preparations from the respective samples by attending pathologists at the respective institutions. The prostatic cell line, LNCaP, was obtained from American Type Culture Collection and cultured as before (Lau and Zhang, 2000). Total RNAs were purified by standard procedures using the Trizol Reagent (Invitrogen-Life Technologies), treated with RNase-free RQ1-DNase (Promega Corp.), extracted with phenol-chloroform, precipitated with ethanol, dissolved in DEPC-treated water and stored at −80 °C.

To determine the transcript profiles of TSPY in both prostate and testis samples, cDNAs were synthesized from purified RNA preparations and amplified with specific primers flanking the open reading frame(s) (ORFs) of TSPY gene (Table 2) using a touchdown PCR procedure, as described before (Lau and Zhang, 2000). Amplified cDNA fragments were subcloned in the pGEM-T Easy plasmid using a TA cloning kit (Promega Corp.). 10–15 individual clones containing a TSPY cDNA insert were randomly selected from each sample, purified and sequenced completely from both directions with an ABI 377 sequencer at the DNA Core Laboratory, Howard Hughes Medical Institute, University of California, San Francisco. The cDNA sequences were analyzed and aligned with previously characterized TSPY cDNA and genomic sequences using the MacVector program. They were further aligned with the working draft sequences of the human genome (April, 2003 freeze) using the BLAT program on the Genome Server at the University of California, Santa Cruz. The BLAT program aligns the cDNAs with the respective genomic sequences revealing the structural genes and exon/intron junctions.

Alternate splice junctions were evaluated by RT-PCR amplification of the respective cDNAs using primers derived from the respective flanking exons (Table 2) and the touchdown PCR technique. These primers were composed of end sequences from the two exons at their respective splice junctions. They were used in combination with either a 5′ (HL3) or a 3′ (HL2) TSPY primer in RT-PCR analysis of RNAs derived from prostatic and testicular samples. Only cDNAs derived from transcripts that had undergone the specific RNA processing could be amplified by PCR with the respective primers. The same primer pairs were also used in secondary PCR amplifications of 1:100 diluted products initially obtained by RT-PCR of prostatic and testicular samples using a set of primers (ATG-5 and S-3) flanking the TSPY ORFs. The amplified products were analyzed by agarose gel electrophoresis. Selected fragments were subcloned into plasmid vector and sequenced with an automated sequencer as above.

Results

TSPY is expressed at elevated levels in tumor cells of prostate cancer

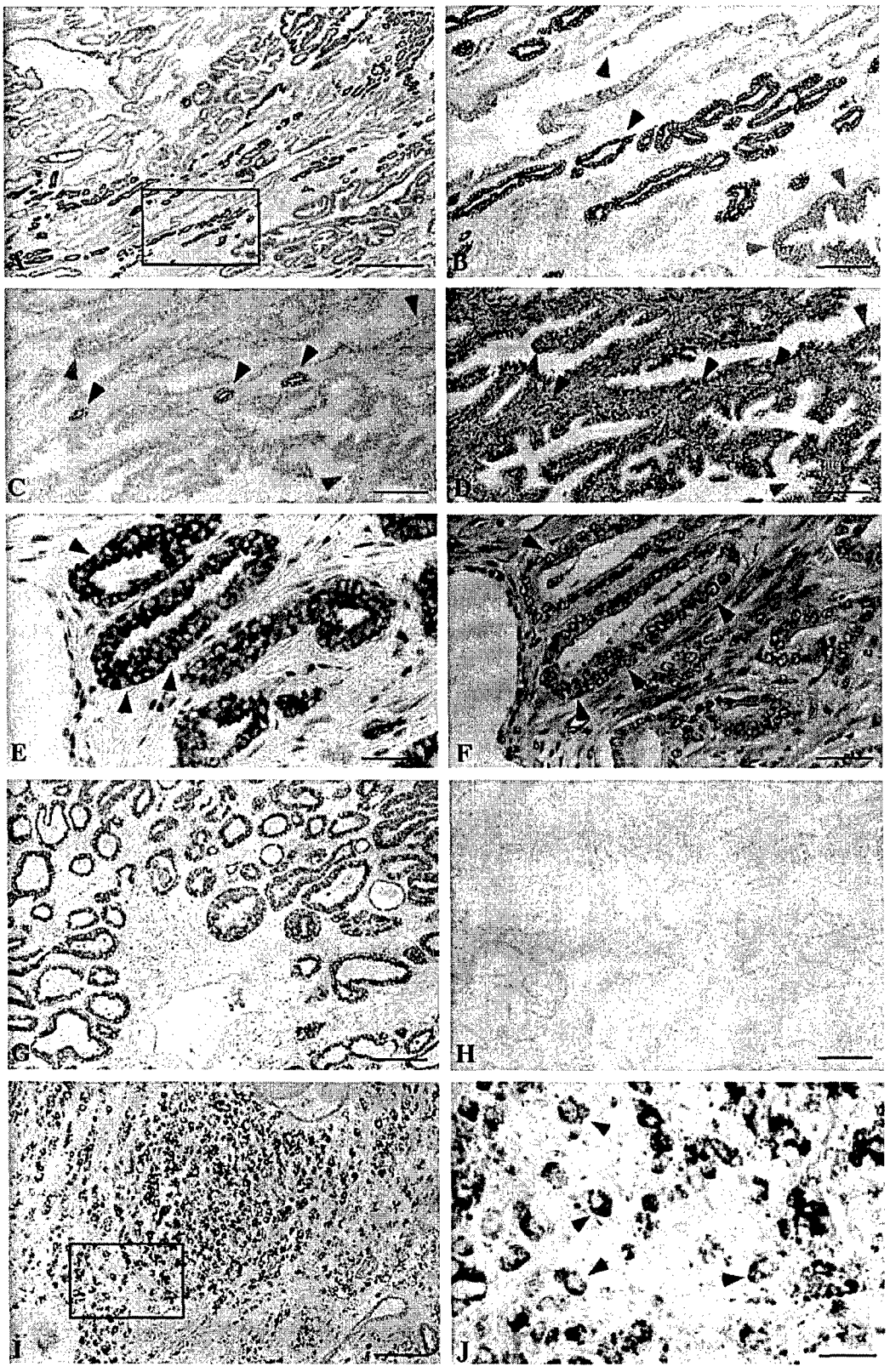
In situ mRNA hybridization was initially used to detect TSPY expression in ten prostate samples containing BPH and/or adenocarcinoma with various Gleason grades (Table 1A). Both antisense and sense biotin-labeled riboprobes were used in the hybridization procedure. We found that incorporating a tyramide amplification step before the substrate development greatly enhanced signal visualization on tissue sections (Speel et al., 1999). Positive signals of varying intensities were observed in all samples with the antisense probe (Fig. 1A, B, C, E, G, I, J) while little or no signals were detected with the sense probe (Fig. 1H). The positive signals were light on normal prostatic structures (Fig. 1B, C, green arrows), moderate on hyperplastic regions (Fig. 1B, orange arrow) and intense on adenocarcinoma (Fig. 1B, C, black arrows). The invading cancerous epithelia (Fig. 1C, black arrows) expressed elevated levels of TSPY mRNA and were clearly discernible among regions of apparently normal morphology (Fig. 1C, D, green arrows). It is interesting to note that such an early invasion of cancerous epithelia was not as readily identifiable on an adjacent section stained only with hematoxylin-eosin (Fig. 1D, black arrows). Under high magnification, the TSPY signals were distributed on the cytoplasm of epithelial cells (Fig. 1E, F, black arrows). TSPY was strongly expressed in adenocarcinoma with increasing grades of malignancy (Fig. 1G, I, J). The signals were also primarily located in the cytoplasm of these tumor cells (e.g. Fig. 1J, black arrows).

To confirm the in situ hybridization results, we extended our study to analyze the TSPY protein expression using a specific polyclonal antibody against a recombinant TSPY protein. Previously, we had used it successfully to detect the TSPY expression in both gonadoblastoma and testicular seminoma sections (Lau, 1999; Lau et al., 2000). Results from this immunohistochemistry study corroborated with those from in situ mRNA hybridization (e.g. Fig. 2A, B and C, D, Fig. 1B, 2J and Fig. 1G, 2E respectively). Again, cancerous cells and/or tumors were clearly visible under low magnification (Fig. 2B). Similar to results from in situ mRNA hybridization, most epithelia

Table 2. Primer sequences used in PCR amplification of cDNAs of TSPY

Primer (abbreviated name)	Sequence
hTSPY-ATG-5 (ATG-5)	5'-ATGCGCCCTGAGGGCTCGCTGA-3'
hTSPY-3 (TSPY-3)	5'-CCATACAATCCACATTTACCCCCTCTTCCTG-3'
hTSPY-S-5 (S-5)	5'-TGGAAGCCCGCGCATGCG-3'
hTSPY-S-3 (S-3)	5'-GACCATGCTGAGTACTGCCGTCCTGCA-3'
PCR4-3 (PCR4-3)	5'-CCTTGAGAATGTTTATTTTTCATTCC-3'
TSPY-Exon1A (1A)	5'-GCACAGGCCTTGGTGGAGCTGGAG-3'
TSPY-Exon1B (1B)	5'-GCACAGGCCTTGCGGGAAAAGATGG-3'
TSPY-Exon1C (1C)	5'-GCACAGGCCTTGATGTCAGCCCTG-3'
Intron4 (Int4)	5'-CGGGAAAGGCCTCATCAGGGCTC-3'
SF-splice (SF)	5'-CATAGGATCTGTCAGGGGACTCAGC-3'
TSPY-HL2 (HL2)	5'-GTCTGCGGCGATAGGCCTCCACTT-3'
TSPY-HL3 (HL3)	5'-TCGGCAGCGGGAAAAGATGGAGCG-3'

Fig. 1. Detection of TSPY expression in prostate samples using in situ mRNA hybridization. (**A–F**) Sample S2249. (**A**) Low magnification view showing a heterogeneous TSPY expression of a prostatic cancer consisting of a mixture of normal (light-staining), hyperplasia (moderate-staining) and tumor (heavy-staining) epithelia. (**B**) Enlargement of the boxed area in (**A**) showing light (green arrow) moderate (orange arrow) and heavy (black arrow) signals. (**C**) Detection of three invading tumor epithelia (black arrows) among the normal prostatic structures (green arrows). (**D**) Hematoxylin-eosin staining of an adjacent section to (**C**). (**E**) High magnification of several tumor epithelia showing cytoplasmic locations of TSPY signals (black arrows). (**F**) Hematoxylin-eosin staining of an adjacent section to (**E**). (**G–H**) Sample S2312. (**G**) In situ hybridization of a tumor using TSPY antisense probe. (**H**) Similar hybridization with a TSPY sense probe on an adjacent section to (**G**). (**I–J**) Sample S3104. (**I**) TSPY mRNA distribution on a highly malignant adenocarcinoma. (**J**) Enlargement of boxed area in (**I**), showing specific expression of TSPY on individual tumor cells. Black arrows indicate examples of cytoplasmic locations of TSPY mRNAs on these cells. The sections were counter-stained with methyl green, except **D** and **F**. The positive signals are brown. Bars represent 800 μm in **A**, 200 μm in **B**, **C**, **D**, **G**, **H** and **I**, and 50 μm in **E**, **F** and **J** respectively.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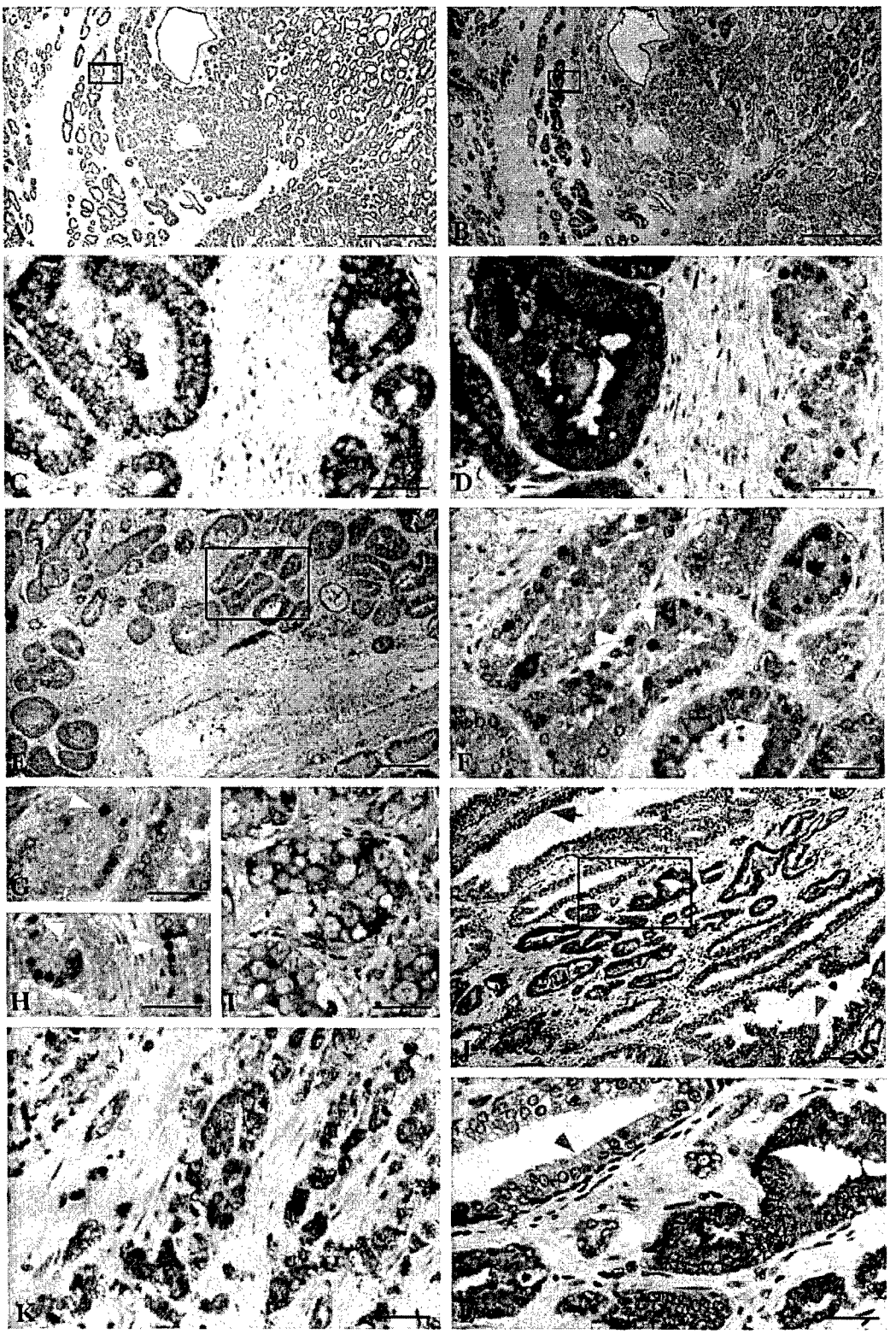
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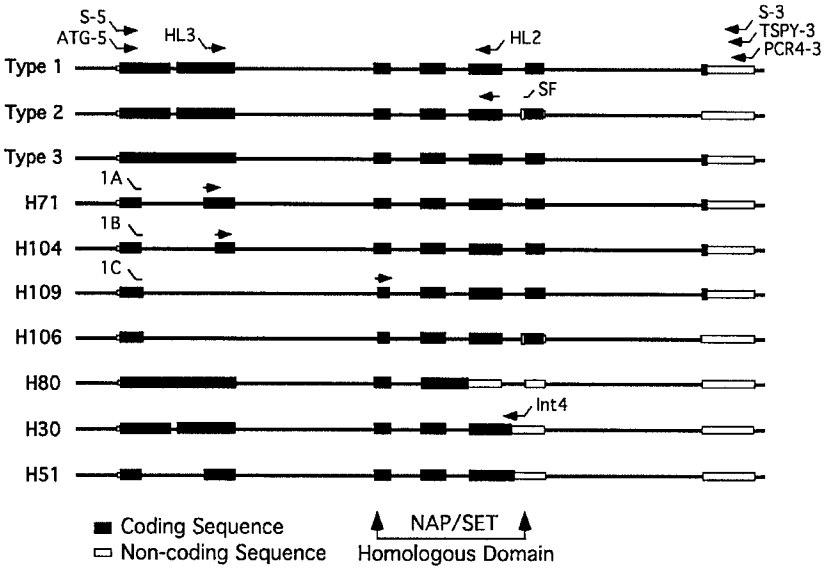

Fig. 3. Diagrammatic illustration of the exon/intron structures and open reading frames of various types of TSPY transcripts. Types 1, 2 and 3 transcripts are previously characterized TSPY transcriptional units. Others are newly identified and characterized in the present study. The positions of the respective primers are as illustrated. The sequences of these primers are listed in Table 2. S-5 and ATG-5 are 5′ primers and S-3, TSPY-3 and PCR4-3 are 3′ primers flanking the ORFs of all known TSPY transcripts. They were used in RT-PCR amplification of cDNAs of the TSPY transcripts from different prostatic and testicular samples. Primers 1A, 1B, 1C, SF and Int4 are specific for the respective splice variants. HL3 and HL2 are 5′ and 3′ primers used in combination with the respective splice junction specific primers in PCR of cDNAs from the tissues. Two vertical arrows indicate the approximate location of the sequence coding for the cyclin B binding domain, similar to those of NAP and SET.

showed varying levels of reactive staining with this TSPY antibody. The signal was low among epithelia of normal morphology (e.g. Fig. 2L, green arrow) and high among those with high-grade cancer (Fig. 2D, E, F, K). Interestingly, some slight differences in immunostaining were observed among adjacent tumor epithelia (Fig. 2D, left and right epithelia) while such differences were not readily detected on in situ mRNA hybridization of a parallel section of the same sample (Fig. 2C). It is uncertain if these minor discrepancies represent true disparity between TSPY mRNA and protein distribution or technical variations between the two techniques used in the studies. Similar to its distribution in gonadoblastoma cells (e.g. Fig. 2I), the TSPY protein was mainly located in the cytoplasm of the prostate cancer cells. However, a significant number of cells showed prominently nuclear staining, especially those of high-grade adenocarcinoma (Fig. 2D, F, G, H, yellow arrows), suggesting that, under certain condition(s), TSPY protein may participate in nuclear activities and/or structural organization of these tumor cells. Immunostaining with specific antibodies against either the proliferating cell nuclear antigen (PCNA) or cyclin B1 showed similar patterns of staining on the same/similar cells/tumors (data not shown). The PCNA was primarily localized on the nuclei while the cyclin B1 signals were detected on both cytoplasm and nuclei of these tumor cells. These observations further suggested that TSPY expression paralleled those of these two proliferating cell markers.

Fig. 2. Corroboration of TSPY expression patterns obtained from in situ hybridization and immunohistochemistry studies. (**A–F**) Sample S2312. (**A**) In situ mRNA hybridization using TSPY antisense probe on a prostate cancer section. (**B**) Immunohistochemistry of an adjacent section using a TSPY specific antibody. (**C**) Enlargement of the boxed area in (**A**). (**D**) Enlargement of the boxed area in (**B**). (**E**) Immunostaining of an adjacent section to Fig. 1G and H. (**F**) Enlargement of boxed area in **E** showing TSPY staining of tumor cells. Although most cells showed cytoplasmic staining, selected cells showed intense staining on their nuclei (yellow arrows), also in **D**, **G** and **H** (Sample S2337). (**I**) Immunostaining of a gonadoblastoma sample (patient 3; Lau et al., 2000) showing prominent cytoplasmic TSPY staining. Blue arrow points to a mitotic cell. (**J**) Heterogeneous expression of TSPY among various epithelia of low/mid grade tumor (Sample S2249), on an adjacent section to that in Fig. 1B. (**K**) Immunostaining of TSPY on a high-grade tumor (Sample S482). (**L**) Enlargement of boxed area in **J**, showing more details of differential staining. Green arrows point to epithelia of apparently normal morphology; orange arrows indicate a potentially hyperplasic epithelium (**J**). Positive signals are brown for in situ hybridization and red for immunohistochemical staining. Bars represent 800 μm in **A** and **B**, 200 μm in **E** and **J**, and 50 μm in **C**, **D**, **F–I**, **K** and **L** respectively.

Differentially processed TSPY transcripts encode a variety of polymorphic proteins

TSPY is a repeated gene with ~35 copies localized on the GBY critical region of the Y chromosome (Skaletsky et al., 2003). Early studies demonstrated that majority of the transcripts are approximately 1.3 kb in size and are derived from 2.8-kb transcriptional units consisting of six exons and five introns (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). So far, three major types of transcripts have been identified and characterized from the testis (Fig. 3). The predominant transcript encodes a protein of 308 amino acids (Schnieders et al., 1996) with a predicted molecular weight of 35.1 kDa. We have designated this transcript as type 1 TSPY transcript. A minor transcript is derived from an alternate RNA processing mechanism utilizing an acceptor site located at 11 nucleotides ahead of exon 5 of the predominant transcriptional unit (Zhang et al., 1992). It encodes a protein with 295 amino acids and a calculated molecular weight of 33.3 kDa. It has been designated as type 2 TSPY transcript. A variant of the predominant transcript harbors an in-frame insertion of an 18-nucleotide repeat within the first exon. It adds six amino acids to the ORF resulting in a protein of 314 amino acids with a calculated molecular weight of 35.8 kDa. It has been designated as type 3 TSPY transcript. All three types of TSPY transcripts maintain the same open reading frame, except where the

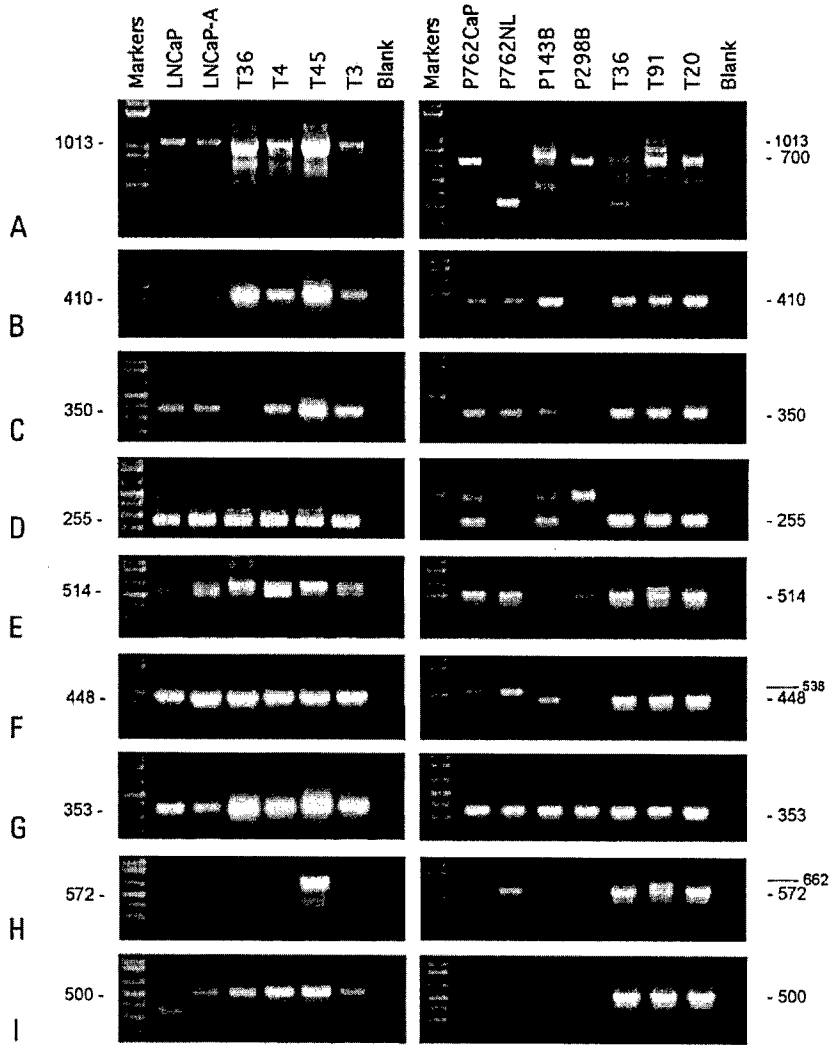

Fig. 4. RT-PCR amplification of TSPY cDNA fragments from prostatic and testicular samples. (**A**) Amplification of cDNA fragments using ATG-5 and S-3 primers flanking the entire ORFs of previously characterized types 1, 2 and 3 transcripts. Primers used in the different RT-PCR amplifications were: (**B**) 1A and HL2; (**C**) 1B and HL2; (**D**) 1C and HL2; (**E**) Int4 and HL3; (**F**) SF and HL3; (**G**) HL3 and HL2; (**H**) 1A and Int4; and (**I**) 1A and SF. See Table 2 for sequences and Fig. 3 for corresponding positions at the transcriptional units. The expected sizes of the respective cDNA fragments are labeled. The numbers in smaller type represent minor fragments amplified with the same primer pairs. See Table 1B for descriptions of the representative prostatic and testicular samples. LNCaP and LNCaP-A were samples from the prostatic cell line, LNCaP. Left panels were derived from direct RT-PCR amplification from respective RNA samples. Right panels were derived from secondary PCR amplification of diluted products obtained initially from RT-PCR of the respective RNA samples using the ATG-5 and S-3 primers. The markers were the 1 kb-plus size markers from Invitrogen-Life Technologies.

changes are present, resulting in slightly polymorphic proteins. They harbor a conserved NAP/SET domain homologous to that shared by some cyclin B binding proteins.

To determine the distribution of the various types of TSPY transcripts in prostatic tissues, a pair of primers (ATG-5 and S-3) flanking the entire ORF of all three types of transcripts were synthesized (Table 2) and used in RT-PCR amplification of the various cDNAs harboring the respective ORFs. Normal and testicular cancer samples were also included in the study. This initial analysis demonstrated a heterogeneous pattern of cDNA products with this primer pair. The predominant bands (e.g. Fig. 4A, left panel) in most testicular samples and the prostatic cell line, LNCaP, seemed to match what were predicted from the previously characterized cDNAs for this gene. Smaller cDNA fragments ranging from 700 to 900 bp were also amplified from these reactions. Significantly these smaller products were especially conspicuous in prostatic samples (e.g. Fig. 4A, right panel). Similar patterns of amplified products were also observed with additional combinations of primer pairs (e.g. Table 2 and Fig. 3, S-5, TSPY-3 and PCR4-3) flanking the entire ORFs. Altering the experimental conditions, such as annealing temperature (up to 68°C), polymerization time (e.g. 1–3 minutes at 72°C) and/or cycle number (e.g. 25–45 with five cycle increments) did not significantly repress the amplification of the smaller cDNA fragments (data not shown). These results supported the postulation that they could indeed be derived from a heterogeneous population of TSPY transcripts in the respective tissues.

To determine the sequences of the cDNA fragments, the amplified products from 12 prostatic and testicular samples (Table 1B) and LNCaP cells were subcloned into the plasmid pGEM-T Easy using the TA cloning technique. Over 120 independent clones were purified and sequenced in both directions using an ABI 377 Sequencer and analyzed with the MacVector program in-house and the BLAT program at the Genome Center Server, University of California, Santa Cruz. Table 3 shows

Table 3. BLAT search results for TSPY transcripts[a]

Transcript	Chromosome	Identity (%)	Strand	Start	End
Type 1	Y	99.6	+	9181262	9184056
		99.5	+	9220141	9222934
		99.5	+	9201607	9204402
Type 2	Y	99.6	+	9181262	9184056
		99.5	+	9220141	9222934
		99.5	+	9201607	9204402
Type 3	Y	99.8	+	9260736	9263548
		99.6	+	9140650	9143463
		99.5	+	9240453	9243266
H71	Y	100.0	+	9140696	9143464
		99.9	+	9220187	9222935
		99.9	+	9201653	9204403
H104	Y	99.8	+	9220173	9222935
		99.7	+	9140682	9143463
		99.6	+	9201639	9204403
H109	Y	99.6	+	9240485	9243267
		99.6	+	9181294	9184057
		99.6	+	9140682	9143464
H106	Y	99.5	+	9240485	9243267
		99.5	+	9181294	9184057
		99.5	+	9140682	9143464
H80	Y	100.0	+	9240499	9243267
		100.0	+	9140696	9143464
		99.9	+	9260782	9263549
H30	Y	99.5	+	9181308	9184057
		99.3	+	9220187	9222935
		99.3	+	9201653	9204403
H51	Y	99.9	+	9220187	9222935
		99.9	+	9201653	9204403
		99.9	+	9140696	9143464

[a] April, 2003 freeze of the human genome sequence assembly at Genome Center Server, University California, Santa Cruz; http://genome.ucsc.edu/

the results of these BLAT searches of the human genome sequences. In general, all transcripts seem to have derived from human repeated TSPY transcriptional units at position Yp11.2. In addition to the three types of previously identified TSPY transcripts, there was a complex array of splice variants of TSPY transcripts (Figs. 3 and 5). They can generally be classified into two categories. The first category concerns the first exon in which a cryptic donor site immediately following the codon encoding amino acid residue #29 was used to splice into three different major acceptor sites within exon 1 and 2 of the type 1 TSPY transcript. The first two variants spliced into sequence preceding amino acid residue #117 and #134 respectively in exon 1 (e.g. H71 and H104 respectively, Figs. 3 and 5) while the third variant spliced into sequence preceding amino acid residue #169 in exon 2 (e.g. H109 and H106, Figs. 3 and 5). The cryptic RNA splices resulted in in-frame deletions of 87, 104 and 139 amino acids from their respective ORFs. These transcripts are designated as variant Exon 1A, Exon 1B and Exon 1C respectively. A small cDNA of ~420 bp was apparently the product of alternative splicing events between the exon 1 cryptic donor site and a cryptic acceptor site in the middle of exon 4. Since this transcript contained no apparent protein-coding ORF, it was not studied any further here. The splice variants of exon 1 were primarily type 1 transcripts (e.g. H71, H104 and H109, Figs. 3 and 5) while a few were rare type 2 transcripts (H106, Figs. 3 and 5). Exon 1A seemed to be the

```
Type 1    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
Type 2    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
Type 3    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H71     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H104     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H109     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H106     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
  H80     1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H30     1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H51     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29

Type 1   51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREAQPRQ  94
Type 2   51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREAQPRQ  94
Type 3   51 GVESEQAALGEEAVLLLDDIMAEVEVVAEVEVVAEEEGLVERREAQPRQ 100
  H71    29 ---------------------------------------------------  29
 H104    29 ---------------------------------------------------  29
 H109    29 ---------------------------------------------------  29
 H106    29 ---------------------------------------------------  29
  H80    29 GVESEQAALGEEAVLLLDDIMAEVEVVAEVEVVAEEEGLVERREAQRAQ 100
  H30    51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREAQRAQ  94
  H51    29 ---------------------------------------------------  29

Type 1   95 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 144
Type 2   95 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 144
Type 3  101 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 150
  H71    29 ----------------------VELEPVNAQARKAFSRQREKMERRRKPH  57
 H104    29 ---------------------------------------REKMERRRKPH  40
 H109    29 ---------------------------------------------------  29
 H106    29 ---------------------------------------------------  29
  H80   101 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPQ 150
  H30    95 QAVPGPGPMTPESALEELLAVQVELEPVNAQSKKAFSRQRENMERTRKPH 144
  H51    29 ----------------------VELEPVNAQARKAFSRQREKMERRRKPH  57

Type 1  145 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
Type 2  145 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
Type 3  151 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 200
  H71    58 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 107
 H104    41 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVGEEKH  90
 H109    29 ------------------------MSALVTDEDEDMLSYMVSLEVEEEKH  55
 H106    29 ------------------------MSALITDEDEDMLSYMVSLEVEEEKH  55
  H80   151 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 200
  H30   145 LDRIGAVIQSVPGFWPNVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
  H51    58 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 107

Type 1  195 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
Type 2  195 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
Type 3  201 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 250
  H71   108 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 157
 H104    91 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 140
 H109    56 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 105
 H106    56 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 105
  H80   201 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITGDRWLPGWVVEGRWWVDHC 250
  H30   195 PVHLYKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
  H51   108 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 157

Type 1  245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 294
Type 2  245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAESPDRSYVRTCGAIPCNTTRG 294
Type 3  251 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 300
  H71   158 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 207
 H104   141 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 190
 H109   106 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 155
 H106   106 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAESPDRSYVRTCGAIPCNTTRG 155
  H80   251 QRDPAPFPQKLLSL                                    265
  H30   245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEVSPHWETN             282
  H51   158 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEVSPHWETN             195

Type 1  295 EEGTETSGDSQLLS  308
Type 2  295 N              295
Type 3  301 EEGTETSGDSQLLSN 315
  H71   208 EEGTETSGDSQLLSN 222
 H104   191 EEGTETSGDSQLLSN 205
 H109   156 EEGTETSGDSQLLSN 170
 H106   156 N              156
  H80   265                265
  H30   282                282
  H51   195                195
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Fig. 5. Protein alignment of type 1, 2, 3 and representative TSPY isoforms predicted from the ORFs of alternatively processed transcripts in prostatic and testicular tissues. H71 and H104 represent proteins encoded by type Exon1A and Exon1B transcripts respectively. H109 and H106 are encoded by type Exon1C transcripts with a type 1 and type 2 carboxyl terminus respectively. H80 is encoded by a type 3 transcript with an unspliced intron 3. H30 is encoded by a type 1 or 2 transcript with an unspliced intron 4. H51 is encoded by a complex transcript involving an Exon1A cryptic splicing at its 5′ end and a skipping of intron 4 at its 3′ terminus. See Fig. 3 for the organization of the corresponding exon/intron junctions and ORFs.

predominant species among these exon 1 variants. The cryptic introns were defined at the donor site by a consensus GT dinucleotide from the valine codon (GTG) at residue #30 while all acceptor sites harbored the AG dinucleotide from a preceding glutamine codon (CAG) at the respective junctions. The calculated molecular weights of the proteins ranged from 18.1 kDa (for H106) to 26.0 kDa (for H71). Significantly, the proteins encoded by the type Exon 1A and 1B transcripts were quite basic with estimated pIs around 8.7 while that encoded by the type Exon 1C transcript would have an estimated pI of 6. The isoelectric points for the TSPY proteins encoded by type 1, 2 and 3 transcripts were around 5. Currently, we have not established a biological function for either these variants or the predominant TSPY protein. However, their differences in protein charge suggest that they might exhibit unique properties. The second category of variant transcripts is a minor one, representing less than 5% of the total TSPY cDNA sequences. It involves the skipping of the small introns, 3 and/or 4, in the RNA processing, resulting in altered ORFs beyond the additional sequence from the intron(s) and slightly different proteins at the carboxyl termini (H80 and H30, Figs. 3 and 5). The calculated molecular weights ranged from 30 to 32 kDa in size and estimated isoelectric points of 4.8. Other rare transcripts might harbor both exon 1 variant(s) and intron skipping in the same transcript (e.g. H51, Figs. 3 and 5). Despite the potential difference(s) in properties, all encoded TSPY proteins, however, still harbor either the whole or a major portion of the NAP/SET domain in their respective ORFs (Fig. 3).

Similar BLAT analysis on the type 3 TSPY transcript suggests that it might also be a product of intron skipping event. The type 3 transcript is extremely rare, representing less than 4% of the total TSPY transcripts. Yet, close to 45% of the TSPY transcriptional units in the human genome database (April, 2003 freeze, Genome Center, University of California, Santa Cruz) contain the 18-bp insertion. Currently, we cannot rule out the possibility that transcriptional units with the 18-bp insert are less active than those without. However, close examination of the 18-bp insert, GTG GAG GTG GTG GCG GAG, suggests that it might be a previously unrecognized small intron harboring the consensus splice junctions of GT and AG at its termini. Hence, it is likely spliced from transcripts originating from transcriptional units containing this 18-bp repeat, resulting in low representation of type 3 transcript in the mature TSPY mRNA population. Alternatively, type 3 transcript can be considered as one that skips this 18-bp intron in the RNA splicing process, as those skipping intron 3 and/or 4, observed in the present study.

To confirm the existence of these splice variant TSPY transcripts, specific primers harboring 12-nucleotide sequences from either ends of the respective splice junctions were used in combination with a common 3′ primer (HL2) in PCR analysis of prostatic and testicular cDNAs (Table 2 and Fig. 3). These cDNAs were synthesized from respective RNA preparations by either reverse transcription alone (Fig. 4, left panel) or RT-PCR amplification with primers flanking the TSPY ORFs (Fig. 4, right panel). To detect type 2 variant and intron-skipping transcripts, additional primers crossing the splice variant exons (i.e. SF) and at intron 4 (i.e. Int4) were used in combination with a 5′ primer (HL3) in similar studies (Table 2 and Fig. 3). Results demonstrated that all variant spliced transcripts were indeed present in both prostatic and testicular samples (Fig. 4B, C, D for Exon1A, 1B and 1C or Fig. 4E, F for type 2 and skipped intron4 transcripts respectively). Further, transcripts with complex splicing patterns, e.g. Exon1A splice and skipped intron 4 (Fig. 4H) or Exon1A and type 2 splices (Fig. 4I), were also detected in these samples. One notable exception was Exon1A and type 2 (SF) splices which was undetectable in prostatic samples (Fig. 4I, right panel), suggesting that such complex RNA processing was either absent in prostatic samples or too infrequent for an effective detection under the experimental conditions. Since specific primers for all variants crossed the respective splice junctions and flanked relatively large introns, successful amplification of the predicted RT-PCR products clearly supports their existence in TSPY transcripts of both prostatic and testicular samples.

Based on the sequencing data, the distributions of transcripts with full length or abbreviated ORFs in testicular and prostatic tissues were somewhat different. For the testicular tissues, 52% of the cDNAs harbored the full length ORFs pertaining to those of the predominant type 1 transcript while the minor type 2 and 3 transcripts represented 11 and 2% of the cDNAs respectively. Approximately 19% of TSPY transcripts were type Exon 1A, 1B and 1C variants. The remaining cDNAs were short ~420-bp non-coding fragments and intron-skipping transcripts with altered TSPY ORFs (e.g. H80 and H30, Fig. 3). For the prostatic samples (including the prostatic cell line), the type 1, 2 and 3 transcripts constituted 37, 3 and 3% respectively of the cDNA population while the exon 1 variants were approximately 34%. The remainder was derived from the intron-skipping rare transcripts (e.g. H80, H30 and H51, Fig. 3) and the non-coding 420-bp cDNA, as observed in the testicular samples.

Discussion

Several genetic studies had clearly demonstrated the existence of the GBY locus, a tumor predisposition or oncogenic locus, on this male-specific chromosome (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). Deletion mapping has assigned the GBY locus to a small region within deletion interval 3 on the short arm and possibly deletion interval 4 proximal to the centromere on the long arm of the Y chromosome (Salo et al., 1995; Tsuchiya et al., 1995). Recent completion of the human Y chromosome sequence suggests that there are ~35 copies of TSPY gene arranged tandemly in 20.4-kb highly (>99%) homologous repeat units located at the GBY critical region (contigs 2 and 3 in Skaletsky et al., 2003). The TSPY cluster comprises of ~700-kb of Yp sequence and is the largest and most homogeneous protein-coding tandem array, so far identified in the human genome. A single-copy TSPY gene is also located distal to this TSPY cluster at Yp (contig 1) and another one is possibly located at the proximal region on Yq (contig 5). Besides the TSPY cluster, no other protein-coding and functional genes were identified within the GBY critical region. These new findings hence further support the notion that TSPY

is the gene for GBY (Lau, 1999). Significantly, others and we had demonstrated high levels of TSPY expression in the tumor germ cells in gonadoblastoma (Hildenbrand et al., 1999; Lau et al., 2000), thereby further strengthening its candidacy as GBY. Interestingly we also observed a similarly high level of TSPY expression in cancerous germ cells at various stages of testicular seminoma (Lau et al., 2000). In a recent survey on the expression of 31 Y chromosome genes in BPHs, prostate cancer samples and prostatic cell lines, we showed that TSPY expression was heterogeneous among these prostatic specimens and was stimulated by androgen in the LNCaP cells (Lau and Zhang, 2000). The present studies localized the TSPY expression on the epithelial cells of the prostatic specimens and demonstrated a preferential elevation of its expression on the adenocarcinoma cells of the prostate. These studies, taken together, not only support the hypothesis that TSPY is GBY but also strongly implicate it to play a role(s) in other male-specific cancers, including prostate and testicular cancers.

Currently, it is still uncertain, how TSPY exerts its oncogenic activities in gonadoblastoma, seminoma and prostate cancer. TSPY has been postulated to serve a normal function of directing the spermatogonial cells to enter meiosis in the testis (Schnieders et al., 1996; Lau, 1999). TSPY harbors a cyclin B binding domain, similar to those of SET oncogene and nucleosome assembly proteins (NAP) (Tsuchiya et al., 1995; Schnieders et al., 1996; Lau, 1999). Some members of this protein family are involved in either regulating or modulating cell cycle progression (Altman and Kellogg, 1997; Shin et al., 1999; Chai et al., 2001; Canela et al., 2003; Pandey et al., 2003). It is uncertain what effects TSPY might have when it is aberrantly expressed in cells and/or tissues incapable of entering male meiosis. Could a quantitative difference in TSPY expression in such tissues/cells, e.g. prostatic cells or female germ cells, potentiate a cell cycle progression and abnormal cell proliferation? The elevated levels of TSPY expression observed in tumor cells of gonadoblastoma, testis and prostate cancers seem to support such a dysregulation hypothesis.

Previous studies had identified several types of transcripts coding for slightly polymorphic proteins (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). Further, base substitutions had also been reported for type 1 TSPY transcripts, involving codon 45, a silent GTG to GTA polymorphism for Val and codon 195, a CCT to CGT polymorphism substituting Pro with Arg (Dechend et al., 2000). These polymorphisms were also observed in our cDNA sequences, including those derived from alternatively spliced transcripts. Further, we had also identified consistent and new amino acid substitutions involving codons 92 and 93 which altered the codons CCC CGA coding for Pro-Arg to codons CGG GCA coding for Arg-Ala at these residues respectively.

The present study demonstrates a more complex pattern of RNA processing, involving cryptic introns and/or alternative donor and acceptor sites of TSPY transcripts. Most alternatively processed TSPY mRNAs maintain ORFs in-frame with that of the predominant type 1 transcript and encode proteins harboring either the whole or part of the conserved cyclin B binding (NAP/SET) domain. The encoded proteins, however, may exhibit significant differences in both the size and properties. Significantly, deletions in type Exon 1A and 1B transcripts result in ORFs encoding relatively more basic proteins than the predominant ones. It will be interesting to determine what effect(s) the deletion(s) might have on the overall function(s) of the TSPY protein. Although we were successful in detecting the expression of the TSPY proteins in prostate cancer using a specific polyclonal antibody, we are uncertain which forms or what expression levels the respective TSPY proteins were in the cancerous cells. Nevertheless, our data raise the possibility that the variant TSPY proteins might indeed possess different properties from those of the predominant form(s). The relative abundance of TSPY exon 1 variant cDNAs from the prostatic transcripts is an interesting observation. In some prostatic and mostly cancerous tissues, the proportion of abbreviated transcripts could be as high as 95% among the cDNA clones examined. Currently, it is uncertain if they play any role in the multi-step process of human oncogenesis. Nevertheless, the present findings on TSPY dysregulation and differential allelic expression have provided a rationale for further investigation on the potential oncogenic or tumor predisposition role of this Y chromosome gene in gonadoblastoma and testicular and prostate cancers.

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Original Paper

Pathobiological implications of the expression of markers of testicular carcinoma *in situ* by fetal germ cells

Friedemann Honecker,[1,2†] Hans Stoop,[1†] Ronald R de Krijger,[1] Yun-Fai Chris Lau,[3] Carsten Bokemeyer[2] and Leendert HJ Looijenga[1]*

[1] *Department of Pathology, Josephine Nefkens Institute, Erasmus MC-University Medical Center Rotterdam, Daniel den Hoed Cancer Center, Rotterdam, The Netherlands*
[2] *Department of Hematology/Oncology, University of Tübingen, Tübingen, Germany*
[3] *Division of Cell and Developmental Genetics, Department of Medicine, VA Medical Center, University of California, San Francisco, California, USA*

*Correspondence to: Leendert HJ Looijenga, Department of Pathology, Erasmus MC-University Medical Center Rotterdam, Josephine Nefkens Institute, Room 430b, PO Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: l.looijenga@erasmusmc.nl

† These authors contributed equally to the work.

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Abstract

Several proteins, such as the placental/germ cell alkaline phosphatases (PLAPs), the stem cell factor receptor c-KIT, and the transcriptional regulator and marker of pluripotency OCT3/4, have been found in both normal immature and malignant germ cells, known as carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU). In the present study, immunohistochemical methods were used to evaluate the expression of these markers in a series of male gonads from fetuses from the second and third trimesters, and neonates. In addition to these markers, the presence of VASA (a protein specific for the germ cell lineage), TSPY (the testis-specific protein, Y-encoded), and the proliferation index (Ki-67 antigen) was analysed. All these proteins are reported to be present both during spermatogenesis and in CIS/ITGCNU. Positive staining for VASA with varying intensity was found in all germ cells, while TSPY was predominantly located in the prespermatogonial cells at all developmental ages. In contrast, the markers PLAP, c-KIT, OCT3/4, and Ki-67 were more frequent at earlier developmental stages and decreased gradually with time, although they could occasionally be detected in germ cells of neonates. These findings are in line with a declining number of gonocytes during fetal development, concomitant with an increase in the number of prespermatogonia. The latter have lost the immature germ cell phenotype. These findings are compatible with the hypothesis that CIS/ITGCNU arises from developmentally arrested germ cells, most likely primordial germ cells/gonocytes, at an early time point during intrauterine development.

Keywords: germ cells; fetal gonads; carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU); immunohistochemistry; developmental arrest

Introduction

In humans and rodents, amongst other species, the embryonic precursors of the gametes are known as primordial germ cells (PGCs) (see ref 1 for a review). These cells are set aside to an extra-embryonic localization early during embryonic development and around the fifth and sixth weeks of human development, they migrate to the area where the genital ridge will be formed [2]. Subsequently, gonadal and sexual differentiation occur in the sixth and seventh weeks [3]. With regard to testicular development, the germ cells at this particular stage are referred to as gonocytes, which are predominantly found in the central areas of the newly formed tubules. Starting around the 14th week of development, these gonocytes gradually migrate towards the tubular periphery. Once the cells are in close contact with the basal lamina of the tubule, they are referred to as prespermatogonia. Maturation is a gradual process; at the 20th gestational week, the fetal testis predominantly contains prespermatogonial germ cells [4]. During the first few years after birth and until puberty, morphological and functional changes occur, including a change from large, immature germ cells (prespermatogonia) to adult type A spermatogonia [5–7]. With the onset of puberty, spermatogonia undergo further spermatogenic maturation and after meiotic divisions, finally produce spermatozoa.

On the basis of multiple findings, it has been hypothesized that CIS/ITGCNU, the common precursor lesion of adult testicular germ cell tumours (TGCTs), originates early during fetal development (see ref 8 for a review). This is illustrated by the presence of a number of markers common to CIS/ITGCNU and immature germ cells, including germ cell/placental alkaline phosphatases (PLAPs), the proto-oncogene receptor c-KIT, and the transcriptional regulator and marker of pluripotency OCT3/4. CIS/ITGCNU cells phenotypically and ultrastructurally resemble PGCs/gonocytes

Table 1. Antibodies (source) and detection method used for immunohistochemistry

Antibody	Company	Code	Pretreatment	Dilution	Secondary antibody (1:200) (biotinylated)	Visualization
PLAP	Cell Marque	CMC203	HIAR*	1:200	Rabbit anti-mouse (Dako E0413)	ABCplx-ap†
c-KIT	Dako	A4502	HIAR	1:500	Swine anti-rabbit (Dako E0413)	ABCplx-ap
OCT3/4	Santa Cruz	sc-8629	HIAR	1:1000	Horse anti-goat (Vector BA9500)	ABCplx-hrp‡
VASA	Provided by D Castrillon [43]		HIAR	1:2000	Swine anti-rabbit	ABCplx-ap
TSPY	Provided by Y Lau [39]		None	1:3000	Swine anti-rabbit	ABCplx-ap
Ki-67	Dako	A047	HIAR	1:50	Swine anti-rabbit	ABCplx-hrp

* Heat-induced antigen retrieval [42].
† ABC complex, alkaline phosphatase, Dako Code: K0391.
‡ ABC complex, horseradish peroxidase, Dako Code: K0377.

[9–11]. Moreover, epidemiological data support the hypothesis that the initiating event of TGCT development occurs during the fetal period [12]. In spite of these data, an alternative origin of TGCTs has been suggested, in which the pachytene spermatocyte, a cell not found during the prepubertal period, has been suggested to be the target of transformation [13].

In order to investigate the origin of CIS/ITGCNU further, we undertook an extensive study of the presence of a number of markers during normal fetal testicular development. This will help us to define further the emerging phenotype of CIS/ITGCNU and shed light on the possible limitations of these markers for early diagnosis of CIS/ITGCNU in high-risk neonates and infants.

Material and methods

Tissue samples

Use of tissues for scientific reasons was approved by an 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).

Human fetal gonads from 27 males from the second and third trimesters after spontaneous or induced abortions (gestational age 15–40 weeks), or from premature and term neonates that had died shortly after birth, and one infant who died from cot death at the age of 6 weeks were obtained from post-mortem sections in our department. Testes were dissected and fixed in 10% formalin and processed into paraffin wax. To ensure satisfactory quality, poor preservation of tissue samples assessed by haematoxylin and eosin staining led to exclusion from this analysis. Cases showing conditions that possibly affect gonadal development, such as trisomy 13, 18 and 21, hydrocephalus, maldeveloped kidneys, or gross intrauterine growth retardation, were excluded from the study. Gestational ages were calculated in relation to the mother's last menstrual cycle and were in accordance with the foot length and the crown–heel length measurements at autopsy, showing a maximum variation of 2 weeks.

Histochemical and immunohistochemical staining

For immunohistochemistry, sections were incubated with the primary antibody overnight at 4 °C (PLAP, c-KIT, TSPY, VASA) or for 2 h at room temperature (OCT3/4, Ki-67). The antibodies used are indicated in Table 1. Sections were counterstained with haematoxylin.

Double staining was performed by using a combination of the same detection method but with different substrates: Fast blue/naphthol ASMX phosphate (F3378 and N500; Sigma, Steinheim, Germany) for blue staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254; Sigma, Steinheim, Germany)/H_2O_2 for red staining, without counterstain. Endogenous peroxidase activity and/or endogenous biotin was blocked using 3% H_2O_2 (for 5 min) and/or a blocking kit for endogenous biotin (Vector Laboratories, Burlingame, CA, USA) to prevent background staining [14].

For quantification, cell numbers showing a positive signal were counted in five to ten cross-sections of seminiferous tubules by two different observers (FH and HS) who were blinded to the gestational age at which the material was sampled.

Results

The presence of a number of well-known and novel markers for CIS/ITGCNU during normal fetal testicular development was studied. The available data on these markers are summarized in Table 2. The results are reported separately for the known CIS/ITGCNU markers (PLAP, c-KIT, and OCT3/4) and the other markers analysed (VASA, TSPY, and Ki-67). Subsequently, the results of double staining are described. Representative illustrations are shown in Figure 1. Figure 2 summarizes the results of each marker for each case individually. For the majority of cases, intra-individual comparison of the expression of CIS/ITGCNU markers (PLAP, c-KIT, and OCT3/4) gave consistent results, reflecting the state of maturation of one particular case. The data for the whole population are depicted graphically in Figure 3 (grey bars) and compared with findings published so far (black bars).

Table 2. Overview of markers for carcinoma *in situ*/intratubular germ cell neoplasia unclassified (CIS/ITGCNU), proliferation, and germ cell-specific factors

Marker/ antigen	CIS/ITGCNU (intensity)	References
Glycogen	+++	Nielsen and Lein, 1974 [20]
PLAP	+++	Beckstead, 1983 [44]; Jacobsen and Norgaard-Pedersen, 1984 [45]
c-KIT	+++	Rajperts-De Meyts and Skakkebæk, 1994 [46]; Strohmeyer *et al*, 1995 [47]
OCT3/4	+++	Looijenga *et al*, 2003 [27]
VASA	+	Zeeman *et al*, 2002 [48]
TSPY	++	Schnieders *et al*, 1996 [38]; Lau *et al*, 2000 [39]
Ki-67	+++	Datta *et al*, 2000 [30]

Immunohistochemical detection of PLAP, c-KIT, and OCT3/4

Positive staining for PLAP, c-KIT, and OCT3/4 was seen specifically in the germ cells, while no staining was present in Sertoli, Leydig, or interstitial cells (see Figure 1).

The highest number of germ cells staining for PLAP was seen in the earliest stages of fetal development examined and decreased continuously throughout the following weeks with advancing gestational age. Within the tubules of earlier stages, PLAP was predominantly detected in gonocytes, and to a lesser extent in prespermatogonia (see Figure 1A). After birth, PLAP could still be found occasionally (see Figure 2A), with maximally one positive cell per visual field. These cells were almost exclusively located in the centre of the tubules. Mainly during the second trimester, a large number of germ cells were positive for c-KIT, which, like PLAP, declined gradually throughout gestation. Again, positive germ cells were detectable at term, albeit at low numbers. Within the tubules, c-KIT was seen in both gonocytes and prespermatogonia (see Figures 1B and 2B).

OCT3/4 resulted in nuclear staining of germ cells at all gestational ages. With advancing gestational age, a constant decrease in cells staining for OCT3/4 was found (see Figure 2C). High numbers with an average of four to six cells per tubule, mainly gonocytes, were seen throughout the first half of the second trimester (Figure 1C). Throughout the second half of the second trimester, the average number of positive cells decreased to less than three cells per tubule. At term, only a few positive germ cells were detectable, mainly located in the centre of the tubules.

An increased number of germ cells positive for PLAP, c-KIT, and OCT3/4 were seen in testes from three second-trimester fetuses with chromosomal abnormalities, ie trisomy 21 and 18: these were not included in the series presented (data not shown). These findings are in line with previous reports [15,16] and support the model that chromosomal abnormalities can interfere with normal germ cell maturation.

Immunohistochemical detection of VASA, TSPY, and Ki-67

VASA-positive germ cells were found at all gestational ages and after birth. Although the staining intensity was variable, prespermatogonia showed consistently stronger staining than gonocytes (see Figure 1D). The number of positive cells per tubule decreased only slightly with gestational age, resulting in a different overall staining pattern compared with the factors described above: VASA was still found in germ cells at term and in neonates (see Figure 2D). Nuclear and cytoplasmic TSPY was predominantly observed in putative prespermatogonia, based on their peripheral localization within the tubules, at all gestational ages. No decrease in staining was found at term or in the first few weeks after birth (see Figure 2E). On average, three to five cells per tubule showed positive staining, most often seen in pairs or groups of germ cells (Figure 1E). In contrast to the other markers described so far, TSPY was not restricted to germ cells, but was also detected in Leydig cells at all ages examined.

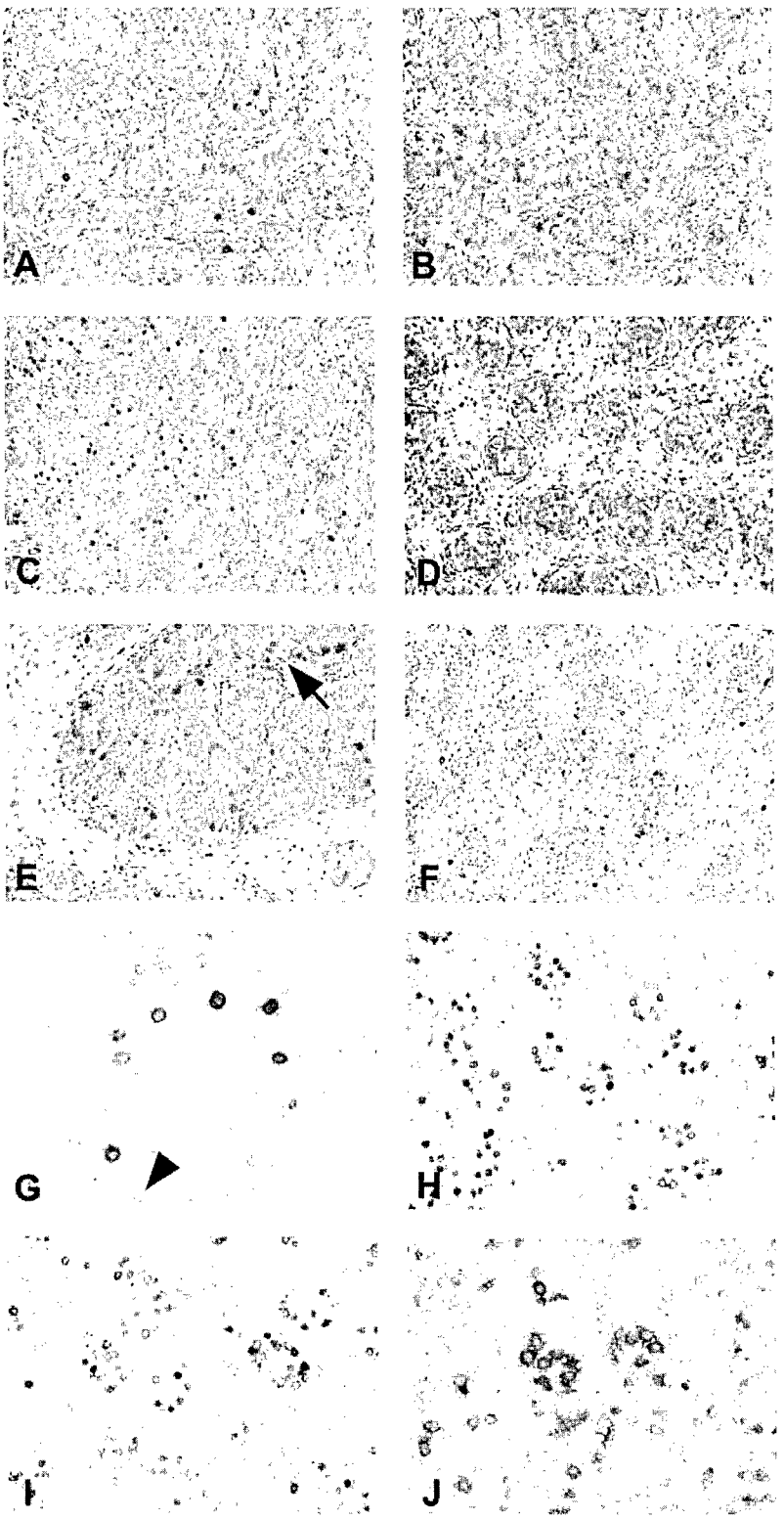
Ki-67 showed nuclear staining in a high number of cells, both within and outside the tubules, throughout the whole period of testicular development investigated. In developing tubules, until 24 weeks of gestational age, Ki-67 was predominantly seen in gonocytes (Figure 1F); after 24 weeks, both basally and centrally located germ cells showed staining in roughly equal numbers. The number of positive cells decreased steadily with advancing age, and perinatally, only a few intratubular cells in the tubules remained positive (see Figure 2F). These were mostly centrally located in the tubules, whereas the majority of prespermatogonia seemed to have entered a quiescent phase at that time.

Results of double staining

Double staining was performed to assess correlations or differences in the presence of various markers in germ cells at different gestational ages. A total of seven cases at 15, 18, 21, 24, 27, 30, and 35 weeks' gestational age were stained for four different combinations: OCT3/4 and PLAP; OCT3/4 and c-KIT; PLAP and c-KIT; and OCT3/4 and VASA (Figures 1G–1J, respectively). The results of all double-staining experiments were in accordance with the single staining results. Staining for OCT3/4 and PLAP revealed that at all stages, a higher number of germ cells were positive for OCT3/4 than for PLAP. PLAP was never detected in germ cells negative for OCT3/4 and the presence of OCT3/4 was more frequently observed at later stages of gestational development, when PLAP was almost undetectable. In contrast, OCT3/4 and c-KIT were found in comparable numbers of germ cells at earlier stages (second trimester), but c-KIT remained positive in more germ cells than OCT3/4 at later stages (third trimester). In agreement with these findings, double staining for PLAP and c-KIT showed that at all gestational ages

more germ cells were positive for c-KIT than for PLAP. PLAP was never detected in germ cells negative for c-KIT, but due to an earlier loss of PLAP, the ratio of cells showing only c-KIT expression versus the number of cells positive for both factors increased from approximately 3 : 1 in the second trimester to approximately 9 : 1 during the third trimester.

An inverse correlation was found between the presence of OCT3/4 and the intensity of VASA. Gonocytes were positive for OCT3/4, but only weakly positive for



VASA during the second trimester, whereas prespermatogonia staining strongly for VASA mostly lacked OCT3/4 (Figure 1J).

Discussion

The aim of this study was to examine the differential presence of a number of factors known to be present in both human fetal germ cells and CIS/ITGCNU, the pre-invasive stage of TGCTs. In addition, we studied a number of factors associated with the proliferation and differentiation of germ cells, allowing us to correlate our findings with the fate of these cells during the second and third trimesters and the first weeks after birth.

Overall, the presence of all CIS/ITGCNU markers decreases during the second and third trimesters (see Figure 2, left panels). Nevertheless, with increasing age, expression was down-regulated differentially. This resulted in a specific expression pattern for each of these factors during fetal male germ cell development. We interpret these data in the context of a gradual process of maturation, extending into the first year of life, which is in accordance with earlier reports [17–20]. The results of the different double-staining experiments indicate that PLAP is the first factor that is down-regulated during the development of normal germ cells, followed by OCT3/4 and finally c-KIT. The fact that CIS/ITGCNU shows high expression of PLAP points towards an early origin of the precursor cell during intrauterine development. In the following paragraphs, the most interesting findings of the individual markers included in this study will be discussed in detail, predominantly in the context of understanding the patho-biological consequences of our findings.

c-KIT is a type III receptor tyrosine kinase, of which stem cell factor (SCF) is the ligand (see ref 21 for a review). The c-KIT/SCF system has been found to be involved in survival and proliferation of migrating germ cells in mice [22]. Our recent finding of the presence of activating mutations affecting one specific site of the receptor in the majority of bilateral TGCTs is in agreement with this model and supports activation of c-KIT as an early initiation event in the pathogenesis of TGCT [23]. Here we show a high presence of c-KIT at the early stages of germ cell development. Its expression generally declines with advancing age during the intrauterine period, but persists throughout the second and third trimesters and to a lesser extent after birth. This is in contrast to earlier reports, in which the authors concluded from a rapid decrease of c-KIT at 10–13 weeks that malignant transformation takes place early during fetal development, possibly even before week 10 [10]. However, more in line with the observation of this study, the detection of c-KIT in germ cells at later stages of development has been described in a recent report [24]. The discrepancies between the different studies might be due to differences in the sensitivity of the antibodies and the detection methods used.

OCT3/4 is a transcriptional regulator, exclusively found in pluripotent human and mouse embryonic stem cells and early germ cells (see refs 25–28 for a review). Recently, it has been described to be present in specific subtypes of TGCTs, including CIS/ITGCNU [27,29], in the context of which it is an informative diagnostic marker [27]. Here we show, in contrast to our earlier more limited study, that OCT3/4 can still be present during the first weeks after birth, albeit at very low frequency. In neonates, OCT3/4 is almost exclusively expressed in gonocyte-like cells located centrally in the tubules, and not in prespermatogonia.

Ki-67 is a nuclear antigen, which is studied in CIS/ITGCNU and TGCTs [30,31]. This antigen is informative in the assessment of proliferation, including germ cells in normal human testis, both during the fetal period and in newborns. Conflicting data on Ki-67 in germ cells at term have been reported [7,32]. To define further the population of proliferating germ cells during the intrauterine period, we assessed the expression of Ki-67 at different gestational ages. Throughout the second trimester, there was a decrease in the number of positive cells, which remained constant at a somewhat lower level at later ages. Interestingly, proliferation was seen in two different populations of germ cells at different time points. Ki-67 was predominantly seen in gonocytes until the end of the second trimester (ie around weeks 24–26). Thereafter, both basally and centrally located cells expressed Ki-67

Figure 1. Results of the immunohistochemical analysis of different markers in fetal germ cells. (A) Fetal testis (15 weeks of development); PLAP (red signal) is seen in gonocytes and prespermatogonia. (B) Same case; c-KIT staining (red signal) is seen in a large number of gonocytes and prespermatogonia. (C) Same case; OCT3/4 (brown nuclear signal). (D) Same case; VASA (red signal). Note the variation in staining, with weaker signal intensity in gonocytes (more centrally in tubules) than in prespermatogonia (on the basal membrane of tubules). (E) Testis of a neonate (6 weeks); TSPY (red nuclear and cytoplasmic signal), mainly in prespermatogonia. Note also the nuclear and cytoplasmic staining of Leydig cells (arrow). (F) Fetal testis, 24 weeks: Ki-67 (brown nuclear signal) is seen both in tubules and in interstitial cells. (G) Fetal testis, 21 weeks: double staining for OCT3/4 (red nuclear signal) and PLAP (blue cytoplasmic signal). OCT3/4 and PLAP are co-expressed in the majority of immature germ cells, and occasional cells are positive for OCT3/4 but negative for PLAP (arrow-head). Note higher magnification. (H) Fetal testis, 15 weeks: double staining for OCT3/4 (blue nuclear signal) and c-KIT (red membranous signal). Note co-expression of OCT3/4 and c-KIT in the majority of germ cells. (I) Fetal testis, 15 weeks: double staining for PLAP (blue cytoplasmic signal) and c-KIT (red membranous signal). Note that co-expression of both markers results in a dark, almost black signal. The majority of germ cells express only c-KIT, whereas only a minority are positive for both markers. (J) Fetal testis, 21 weeks: double staining for OCT3/4 (red nuclear signal) and VASA (blue cytoplasmic signal). Germ cells showing a strong intensity for VASA are negative for OCT3/4 and are more often found on the basal lamina of the tubule. Note higher magnification

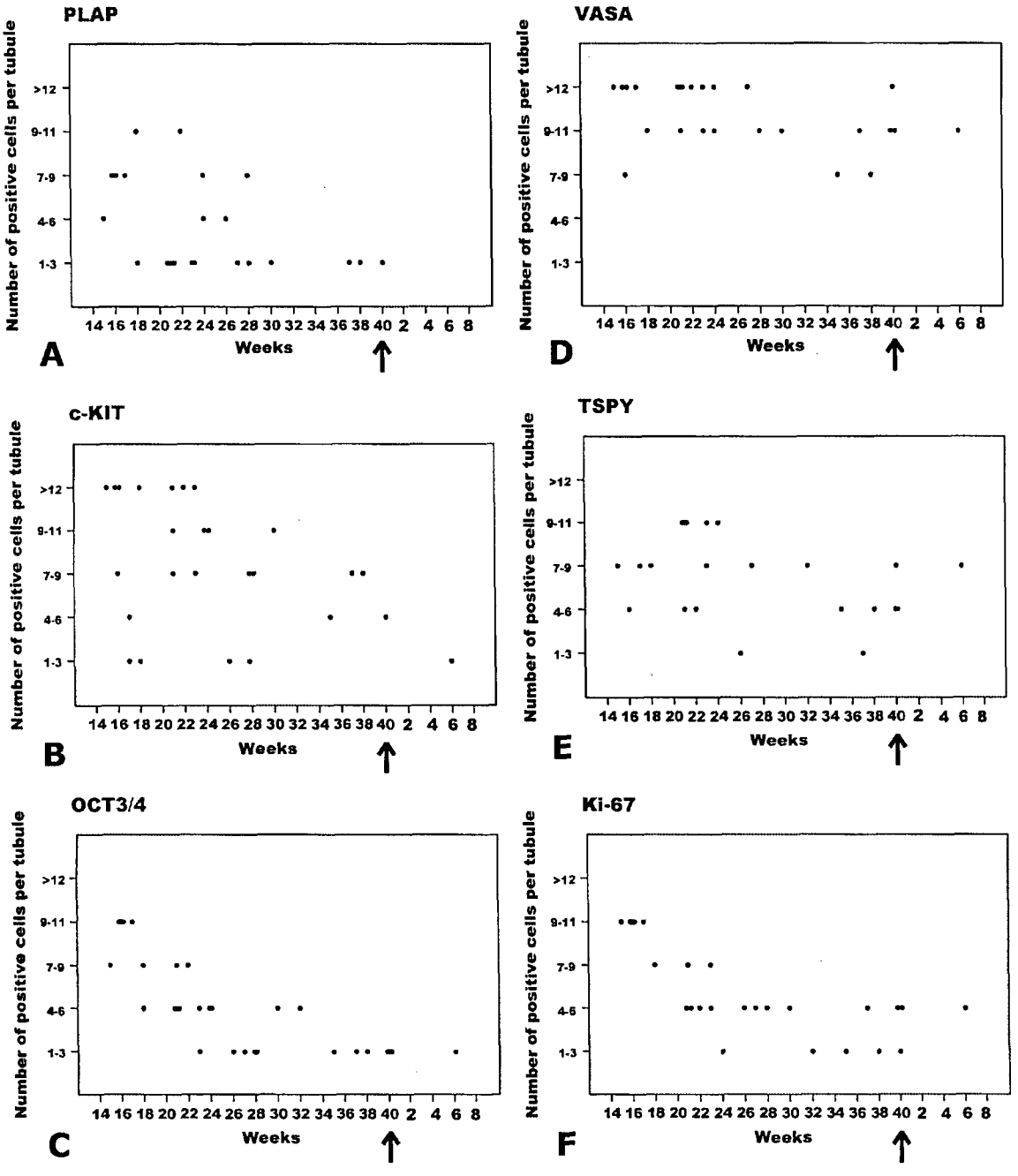


Figure 2. Absolute numbers of cells per tubule showing positive immunohistochemical staining for the markers at different gestational ages (weeks). The earliest case studied in this series was 15 weeks; the oldest was a neonate of 6 weeks. The arrow indicates the time of birth (median 40 ± 2 weeks). The left panels (A–C) show markers for CIS/ITGCNU; the right panels (D–F) illustrate factors associated with germ cell differentiation and proliferation. Each black spot represents one case

in roughly equal numbers, suggesting that, in addition to gonocytes, the pool of more differentiated prespermatogonia also expands during the late second and third trimesters, extending into the first weeks after birth.

Human TSPY, the testis-specific protein, Y-encoded, gene, is organized as a repetitive gene family mapped to the critical region of the gonadoblastoma (GBY) [33–35] locus on the short arm of the Y chromosome [36,37]. It is mainly found during early spermatogenesis and has been suggested to play a normal role in spermatogonial proliferation and an oncogenic role in early germ cell tumourigenesis [38,39]. Expression of *TSPY* sequences has been found by RNA analysis in prenatal and adult testes [37,40,41]. To our knowledge, we describe for the first time the presence of TSPY protein in human germ cells during male gonadal development. Staining was often seen

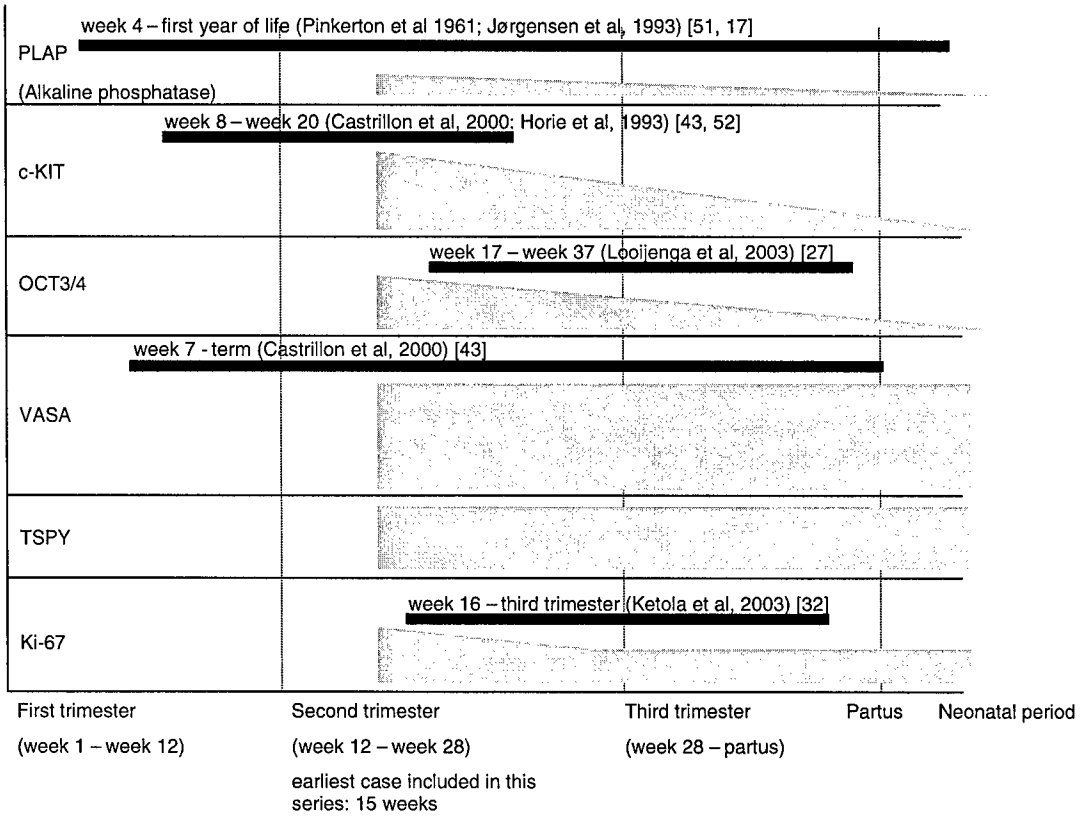


Figure 3. Overview of the data currently available from the literature on the expression of different antigens in germ cells during intrauterine development (black bars) compared with the findings of this study (grey bars). The sizes of the grey bars schematically represent the frequency of germ cells expressing the individual factor at different developmental ages

in groups of prespermatogonia throughout the second and third trimesters and in neonates. Although little is known about the function of this protein, a role in the regulation of the proliferation of germ cells is tempting, as has been suggested before [39].

In summary, our data indicate that during the second trimester, gonocytes are positive for a number of CIS/ITGCNU markers, such as OCT3/4, c-KIT, and PLAP. During the process of maturation towards prespermatogonia, these cells gradually lose these early markers. During further development, there is a relative decrease in the number of gonocytes compared with more mature prespermatogonia. Interestingly, the time point at which more and more germ cells become attached to the basal lamina of the tubule (between weeks 20 and 24) coincides with an overall decrease in Ki-67 expression, down-regulation of the CIS/ITGCNU markers, and an increase in VASA staining intensity. This suggests a preference for differentiation over proliferation at the transition from gonocytes to prespermatogonia during the second trimester and indicates that attachment to the basal membrane could be important for germ cell maturation. This model is supported by the results of the double-staining experiments for OCT3/4 and VASA, where gonocytes in the more central areas of developing tubules show expression of OCT3/4, but low staining intensity for VASA. Although de-differentiation and consecutive re-expression of early markers cannot be ruled out as a mechanism for the development of CIS/ITGCNU, our data are in line with the model of a maturation arrest of immature germ cells as one of the first pathogenetic hits in the development of TGCTs.

Two observations from the study presented are particularly noteworthy. First, the presence of markers such as PLAP, c-KIT, and OCT3/4 is not restricted to the early stages of germ cell maturation, but extends well into the second and third trimesters and can, in fact, still be found in neonates. Therefore, in contrast to the situation in the testes of adolescents and adults, these markers can be unreliable for the detection of CIS/ITGCNU in very young children. This adds further evidence to the notion that testicular biopsy is of limited value in this age group [49,50]. Second, gonocytes that are positive for these markers at the later stages of normal development are hardly ever found on the basal membrane of the seminiferous tubules, but are localized more centrally. This distinguishes them from CIS/ITGCNU cells, which are always in contact with the basal membrane and phenotypically and ultrastructurally resemble prespermatogonia [11]. This indicates that CIS/ITGCNU cells, possibly due to a maturation arrest, show a certain marker profile that

normal germ cells in this localization have already lost. However, while retaining features of immature germ cells, CIS/ITGCNU cells also show some potential to develop along the germ cell lineage, as is documented by the presence of VASA and TSPY in these cells.

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