Cancer/testis (CT) antigens, carcinogenesis and spermatogenesis

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During spermatogenesis, spermatogonial stem cells, undifferentiated and differentiated spermatogonia, spermatocytes, spermatids and spermatozoa all express specific antigens, yet the functions of many of these antigens remain unexplored. Studies in the past three decades have shown that many of these transiently expressed genes in developing germ cells are proto-oncogenes and oncogenes, which are expressed only in the testis and various types of cancers in humans and rodents. As such, these antigens are designated cancer/testis antigens (CT antigens). Since the early 1980s, about 70 families of CT antigens have been identified with over 140 members are known to date. Due to their restricted expression in the testis and in various tumors in humans, they have been used as the target of immunotherapy. Multiple clinical trials at different phases are now being conducted with some promising results. Interestingly, in a significant number of cancer patients, antibodies against some of these CT antigens were detected in their sera. However, antibodies against these CT antigens in humans under normal physiological conditions have yet to be reported even though many of these antigens are residing outside of the blood-testis barrier (BTB), such as in the basal compartment of the seminiferous epithelium and in the stem cell niche in the testis. In this review, we summarize latest findings in the field regarding several selected CT antigens which may be intimately related to spermatogenesis due to their unusual restricted expression during different discrete events of spermatogenesis, such as cell cycle progression, meiosis and spermiogenesis. This information should be helpful to investigators in the field to study the roles of these oncogenes in spermatogenesis.

Introduction

Spermatogenesis is the major physiological event that occurs in the seminiferous tubules, the functional units of the testis that produce spermatozoa in men as well as in rodents.¹ Each man produces an upward of 100 million spermatozoa each day since

puberty at ~12–14 years of age through his entire adulthood under the influence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) released from the pituitary gland.^{1–4} FSH exerts its effects on Sertoli cells in the seminiferous epithelium to maintain spermatogenesis via FSH receptors which are restricted to the Sertoli cell in the mammalian body.^{1,5–7} On the other hand, LH binds to the LH receptors residing in Leydig cells in the interstitial space between seminiferous tubules to induce steroidogenesis for the production of testosterone and estradiol-17 β .^{2,3,8–12} Since the release of these two pituitary hormones that regulate spermatogenesis is under the influence of the hypothalamus, the intricate hormonal and functional relationship between the hypothalamus, pituitary gland and the testis is known as the hypothalamic-pituitary-testicular axis. This axis is also the target of male hormonal contraception by using testosterone- or testosterone-progesterone-based injectables, pills, patches, and/or implants.^{13–18} While the hormonal events along the hypothalamic-pituitary-testicular axis and the detailed morphological events that occur in the seminiferous epithelium known as the seminiferous epithelial cycle have been eminently described and reported almost 70–80 years ago,^{2,3,10,12,19–23} the molecular and biochemical mechanism(s) that regulate spermatogenesis in the seminiferous epithelium remain largely unexplored. As a result, this poses major hurdles in developing safe and reliable male contraceptives in particular non-hormonal products.^{24–27}

While spermatogenesis is composed of a series of highly complex cellular events, it can be broadly divided into several discrete events: (1) renewal of spermatogonial stem cells and spermatogonia via mitosis, (2) proliferation (via mitosis) and differentiation of spermatogonia through type A and type B, and eventually preleptotene spermatocytes, (3) meiosis, (4) spermiogenesis (transformation of round spermatids to elongated spermatids and spermatozoa) and (5) spermiation (the release of sperm from the epithelium into the tubule lumen).^{1,21,28,29} **Figure 1** depicts the cross-section of an adult rat testis applicable to other mammalian testes, showing sections of several seminiferous tubules at various stages of the seminiferous epithelial cycle of spermatogenesis. This figure also illustrates germ cells at different stages of their development are intimately associated and supported by Sertoli cells, which, in turn, constitute the seminiferous epithelium overlying the tunica propria. The tunica propria is composed of an acellular zone of basement membrane (a modified form

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Figure 1. Morphological and cellular features of spermatogenesis in the mammalian testis. The anatomical features of the rat testis shown here (A) share many of the feature similar to other mammalian testes including humans, and the schematic drawing shown in (B) depicts the major cellular events of spermatogenesis, namely spermatogonia/SSC self-renewal via mitosis and differentiation to type B spermatogonia, which in turn transform to preleptotene spermatocytes. Preleptotene spermatocytes are the germ cells residing in the basal compartment traverse the blood-testis barrier (BTB) at stage VIII of the epithelial cycle that enter the adluminal (apical) compartment while differentiate into leptotene, zygotene and then pachytene spermatocytes in a process known as cell cycle progression. Diplotene spermatocytes eventually enter meiosis I to be immediately followed by meiosis II at stage XIV as shown in (A) to form haploid round spermatids (step I spermatids). Spermatids undergo spermiogenesis to form elongating and elongated spermatids involving 19 and 16 steps in rats and mice, respectively. Elongated spermatids transform into spermatozoa following the shredding of the residual body to be phagocytosed by the Sertoli cells to allow the release of sperm at spermiation. The fine morphological features of some of these germ cells are also depicted in (B). It is noted that the seminiferous epithelium is resting on the tunica propria, which is composed of an acellular zone namely basement membrane (a modified form of extracellular matrix) and type I collagen layer, and a cellular zone of peritubular myoid cell layer and the lymphatic microvessel. It is noted that the seminiferous epithelium is composed of only Sertoli and germ cells without the presence of any blood vessels and nerve fibers since all microvessels are restricted to the interstitial space between seminiferous tubules. The BTB also physically divides the seminiferous epithelium into the basal and adluminal (apical) compartments. Different cancer/testis (CT) antigens are expressed throughout spermatogenesis with unique patterns of cellular expression among different types of germ cells. PLS, preleptotene spermatocyte; RS, round spermatid.

of extracellular matrix which is mainly composed of laminin and type IV collagen^{30,31}), and type I collagen layer, underneath of which is a cellular zone composed of peritubular myoid cell layer and lymphatic vessel (Fig. 1). In addition, the seminiferous epithelium is anatomically divided into the apical (adluminal) and basal compartments by the blood-testis barrier (BTB), which is located near the basement membrane in the mammalian testis. The BTB is formed by specialized junctions at the adjacent Sertoli cells in the seminiferous epithelium, which are

composed of coexisting tight junction (TJ), basal ES [basal ectoplasmic specialization, a testis-specific atypical adherens junction (AJ)] type³¹⁻³³, gap junction and desmosome.^{32,34,35} This ultrastructure segregates the events of meiosis I and II, spermiogenesis and spermiation from the host animal's immune system, so that all the post-meiotic spermatids are developed in a specialized microenvironment, namely the adluminal compartment, behind the BTB. The BTB, together with the secretory products from Sertoli cells, such as interleukins and interferons,³⁶⁻³⁸ thus confer

an immune privilege status to the testis. This barrier is physiologically significant since during each of the five different cellular events that encompass spermatogenesis, numerous germ cell-specific antigens express transiently, some of which are residing on the cell surface. Thus, the BTB “seals” the exposure of these antigens to the host immune system to avoid the production of anti-sperm antibodies, which, if occurs, will lead to infertility in men. Additionally, Sertoli cells also secrete humoral factors (e.g., interleukins, interferons and cytokines) to confer an immune privilege status to the testis.^{35,39-41}

Interestingly, studies in past 15 years have shown that developing germ cells express a large number of genes, many of which were subsequently shown to be oncogenes and/or proto-oncogenes, which are either not expressed or expressed at exceedingly low level in normal mammalian cells unless they are induced and/or transformed to undergo carcinogenesis. However, normal testis per se is not a tumor, and many of the developing germ cells, including spermatogonia, spermatocytes, round and elongating spermatids only express these genes transiently, such as at specific steps of spermatids during spermiogenesis and differentiating spermatogonia, as well as differentiating spermatocytes that are preparing for meiosis. Also, greater than 75% of the developing germ cells undergo apoptosis to avoid overwhelming the limited number of Sertoli cells (estimated to be ~25–40 million per testis in the rat.⁴²⁻⁴⁴ Sertoli cells cease to divide by about 17 days postpartum (dpp) in rats⁴⁵ even though recent studies have shown that adult mouse and human Sertoli cells can become proliferative when cultured in vitro under certain conditions^{46,47}). Furthermore, some of the transiently expressed proteins have crucial functions to spermatogenesis (e.g., cell adhesion) and reproductive processes (e.g., sperm-egg interactions) (see sections below), yet they were subsequently found to be expressed abundantly only in metastatic or tumor cells but not in any other normal cells and/or tissues besides the testis and the placenta (and the ovary in some cases). Thus, these antigens are known as cancer/testis (CT) antigens.^{48,49} In short, the testis is not a tumor per se, but it does express many of the oncogenes that are restricted to tumorigenesis and metastasis. Herein, we review some of the recent findings regarding the roles of CT antigens in spermatogenesis and carcinogenesis. A better understanding on this group of proteins will not only unfold their roles in the seminiferous epithelial cycle of spermatogenesis but will also provide us with a clearer insight into how to target immunotherapy towards CT antigens in cancer.

Cancer/Testis (CT) antigens

The term “Cancer/Testis (CT) antigen (or CTA)” was first used in 1997.⁵⁰ CT antigens, also known as cancer germline antigens, refer to a growing list of antigens that were initially discovered in the 1980s–1990s,⁵⁰⁻⁵⁷ which specifically expressed in various tumors of different histological origins. CT antigens are absent in normal somatic cells in humans and rodents but expressed only in male germ cells (such as spermatogonial stem cells, spermatogonia, spermatocytes, spermatids and spermatozoa during spermatogenesis) in the testis (but not Sertoli and/or Leydig cells).⁵⁸⁻⁶³

Table 1. Members of the CT antigen family*

CT antigens	Alias	Genes	Chromosomes
Family			
MAGE			
MAGE-A	CT1	15	Xq28
MAGE-B	MAGE-Xp; DAM-6, -10	17	Xp21
MAGE-C	CT7; CT10	7	Xq26-27
MAGE-D	magphinin, NRAGE	5	Xp11
Necdin		1	15q11.2-12
GAGE/PAGE/XAGE			
GAGE	CT3	9	Xp11.4-11.2
PAGE	CT16; GAGE-B, -C	5	Xp11.23
NY-ESO-1/LAGE			
NY-ESO-1	CTAG-1; CAG-3	1	Xq28
LAGE	CTAG-2	1	Xq28
SSX			
SSX	HOM-MEL-40; CT4	5	Xp11.2
SPANX			
SPANX	Ctp11	6	Xq26.3-27.1
PIWI			
Piwi2			
PL2L			
Non-familial CT antigens			
SCP-1	HOM-TES14; CT6		1p13
OY-TES-1	sp32; CT14		12p13.32
SP-17			

*This table was prepared based on earlier reviews in references 59, 66 and additional references as detailed in the main text. This Table is not intended to be exhaustive, it only lists selected CT antigens pertinent to spermatogenesis based on their unique pattern of distribution (based on studies using immunohistochemistry, northern blotting or PCR) among selected germ cell types in the seminiferous epithelium of human testes from normal men.

In some cases, CT antigens are also expressed in ovary and in the placenta (usually in trophoblast cells), such as NY-ESO-1, a CT antigen originally identified from esophageal squamous cell carcinoma of a 58-year-old female cancer patient.^{50,58-63} More importantly, the genes of CT antigens are activated and highly expressed in a number of malignant tumors and they are antigenic in tumor-bearing patients.^{50,60} Thus, many CT antigens were identified by using sera of cancer patients to screen human testis cDNA library or recombinant proteins.^{50,59,64,65}

CT antigens are broadly divided into those that are encoded by genes residing on the X chromosomes, the X-CT antigens, and those that are not, the non-X-CT antigens.⁵⁹ However, X-CT antigens represent more than half of all CT antigens identified to date (Table 1). They are often organized in well-defined clusters to constitute multigene families along the X-chromosome, where different members of a single family are arranged into complex direct and inverted repeats.^{48,59,66} The genes encoding non-X-CT antigens, however, are distributed throughout the genome and

are mostly single-copy genes⁵⁹ (Table 1). While the functions of many of these CT antigens remain unknown, recent studies showed that these proteins are likely involved in cell cycle progression/regulation, transcriptional control, cell survival and apoptosis. Studies on the expression of CT antigens have also shown that epigenetic events, such as DNA methylation, are the primary mechanism regulating the expression of CT antigens in germ cells and transformed cells.⁴⁸ Also, CT antigens (e.g., MAGE proteins) are frequently mutated in cancers,⁶⁷ and many CT antigens were identified by using sera of cancer patients to screen human testis cDNA library or recombinant proteins,^{50,59,64,65} illustrating they are highly antigenic. In light of their restricted expression in tumor and cancer stem cells and their antigenic properties, several CT antigens have been the targets of cancer immunotherapy (e.g., vaccine therapy) and some of which are biomarkers of various malignancies, such as ovarian cancer, cervical cancer, breast cancer, lung cancer, urinary bladder cancer, osteosarcoma and testicular cancer.^{48,60,68-74} Unfortunately, some of the recently completed clinical trials revealed that even though the immunotherapy that targets CT antigens could shrink the tumor, it failed to increase the survival rate of the treated patients versus patients that received placebo,^{48,75} illustrating the complexity of CT antigens.⁴⁸ At present, there are ~70 families of CT antigens and about 140 members have been identified, with their functions largely unknown.^{48,60} Since each cancer type is associated with multiple highly expressed CT antigens, an effective vaccine may require the presence of multiple CT antigens. In this review, we summarize some of the latest findings in the field regarding the roles of several best studied CT antigens during tumorigenesis. These selections were based on their intrinsic activities, mode of actions and/or immunohistochemical localization patterns in human testis, illustrating they are intimately related to spermatogenesis, such as their association with spermatogonia, spermatocytes, post-meiotic spermatids and spermatozoa. These findings thus illustrate the possible roles of CT antigens during spermatogenesis, and functional experiments can be designed in future studies (Fig. 2).

CT Antigens: Their Roles in Spermatogenesis and Tumorigenesis

Sperm protein 17 (SP17). SP17 was originally identified as an autoantigen in the mouse testis. It is highly expressed in mouse⁷⁶ and human⁷⁷ spermatozoa and is restricted to the tail (in particular the fibrous sheath⁷⁸) and over the acrosomal region of the head of mature spermatozoa⁷⁶ (Fig. 2). The primary sequence of SP17 is composed of three domains: (1) a conserved N-terminal domain that shares homology with type II α regulatory subunit of protein kinase A (PKA-RII α) that binds A-kinase anchoring protein 3 (AKAP3) in sperm flagella with high affinity (note: AKAP3 is involved in regulating human sperm motility following its coupling to the regulatory subunit of PKA.^{79,80} AKAP3 is also a CT antigen restricted to human testis in normal tissue but highly expressed in tumors and its expression is correlated with a poor prognosis and low survival rate in ovarian cancer patients⁶⁰) (2) a central sulfated carbohydrate (heparin)-binding

domain which contains two putative heparin-binding motifs (BBXB, where B is any basic amino acid, X is any amino acid) of KREK (residues 49–52) and KKIR (residues 131–134), and (3) a Ca²⁺/calmodulin binding domain near its C-terminus.⁸¹⁻⁸³ Its functions remain unknown except it was implicated to be: (1) involved in sperm-egg interactions between the acrosome-reacted sperm and zona pellucida of the ovum during fertilization^{84,85} in light of its restricted localization at the acrosome of normal and capacitated spermatozoa undergoing acrosome reaction⁷⁶ and (2) involved in cell adhesion and/or cell migration possibly through the interaction of its heparin-binding motifs with heparan sulfate in extracellular matrix (e.g., in malignant lymphocytes).⁸⁶ Also, Sp17 can serve as a scaffolding protein to form a PKA-independent AKAP protein complex via its AKAP-binding motif to recruit additional signaling proteins to specific cellular site(s) in both germ cells and malignant cells.⁸⁷

A mouse homolog of SP17 was found to be highly expressed in metastatic cell lines derived from a mouse model of squamous cell carcinoma but not in the nonmetastatic parental line,⁸⁸ illustrating the potential of SP17 as a therapeutic target. Subsequent studies have shown that SP17 was highly expressed in cancers of unrelated histological origins, such as multiple myeloma, ovarian cancer, brain tumors, esophageal cancer, ovarian cancer, endometrial cancer and cervical cancer.^{73,89-94} Thus, gynecological cancers appeared to be the best candidates for SP17 targeted immunotherapy because it is virtually absent in normal tissues in women.⁹⁵ Unfortunately, subsequent studies have identified that SP17, unlike most CT antigens, is expressed also in somatic cells including the ciliated epithelia of the respiratory airways and reproductive tract of both men and women,⁹⁶ synoviocytes from patients of rheumatoid arthritis,⁹⁷ and melanophages of cutaneous melanocytic lesions.⁹⁸ These findings thus raise a question as to whether SP17 can be a target for immunotherapy due to its wide-spread presence in other epithelial cells. Thus, no clinical trials have been performed using SP17 as an immunotarget. Interestingly, in a mouse model of ovarian cancer, vaccination of recombinant SP17 protein was found to provide long-term protective effect on reducing the tumor size and controlling tumor growth in mice.⁹⁹ Nonetheless, a better understanding of SP17 in the testis, in particular in spermatogenesis, such as its involvement in spermiogenesis, may offer hints on the roles and functions of this protein in tumorigenesis and/or metastasis.

NY-ESO-1. NY-ESO-1, also known as CTAG1B (cancer/testis antigen 1B), is a 22 kDa protein that was originally found and isolated from a New York City 58-year-old female cancer patient of esophageal squamous cell carcinoma.⁵⁰ NY-ESO-1 together with LAGE-1 (also known as CTAG2, cancer/testis antigen 2¹⁰⁰ which shows no expression in normal tissues other than the testis⁶⁰) constitute the NY-ESO-1/LAGE family of CT antigens^{59,66} (Table 1). NY-ESO-1 is one of the best studied CT antigens and its mRNA is highly expressed in the testis [restricted mostly to spermatogonia and primary spermatocytes but not in post-meiotic spermatids nor Sertoli cells in humans^{101,102} (Fig. 2)], ovary (but not in any normal tissues) and human melanoma, breast cancer, bladder cancer, gastric cancer, lung cancer, sarcoma, ovarian cancer, prostate cancer and hepatocellular carcinoma.^{50,103,104} Due

Figure 2. A schematic drawing illustrating the restricted expression of different CT antigens in germ cells during spermatogenesis. The part on the right depicts different germ cell types during spermatogenesis, and the left part illustrates the unique expression patterns of seven different CT antigens in different germ cell types, illustrating their possible involvement in different phases of spermatogenesis. These CT antigens, besides their restricted expression in the testis but not other normal human cells/tissues, they are highly expressed in cancer cells. As such, many of these antigens are the targets of immunotherapy and vaccine development for cancer treatment. Yet their functional significance in the testis remains largely unexplored.

Table 2. Selected clinical trials of immunotherapy by targeting CT antigens*

Targeted CT antigen	Sponsor	Tumor histotype	Phase	Status	Identifier
NY-ESO-1 vaccine	ImmunoFrontier, Inc.	Solid tumors (e.g., melanoma, breast cancer, ovarian cancer, sarcoma and other expressing NY-ESO-1)	I	Ongoing 05/2011–12/2012	NCT01234012
NY-ESO Phase I Study for Prostate Cancer	Baylor College of Medicine	Prostate Cancer	I	Ongoing 06/2006–12/2016	NCT00711334
Infusion of genetically Engineered NY-ESO-1 T cells	University of Pennsylvania	synovial sarcoma	I	Ongoing 03/2011–03/2028	NCT01343043
MAGE-A3 Protein + AS15 for multiple myeloma patients undergoing autologous stem cell transplant	Ludwig Institute for Cancer Research/GlaxoSmithKline	Multiple Myeloma	I	Ongoing 06/2011–04/2014	NCT01380145
MAGE-A3 and NY-ESO-1 immunotherapy in combination with DTPACE chemotherapy	Myeloma Institute for Research & Therapy, University of Arkansas	Multiple myeloma	I	Ongoing	NCT00090493
MAGE-A3/recombinant protein immunotherapeutic vaccine	GlaxoSmithKline	Metastatic cutaneous melanoma	I	Completed, no study results posted	NCT00706238
Melanoma Vaccine	Maria Sklodowska-Curie Memorial Cancer Center	Melanoma vaccine in patients with Stage III disease after lymph nodes removed	I & II	Completed, no study results posted	NCT01082198
MAGRIT MAGE-A3 antigen Specific cancer immuno-therapeutic (ASCI)/MAGE-A3 Vaccine	GlaxoSmithKline	MAGE-A3 positive non small cell lung cancer ($n = 2,270$ patients)	III	Ongoing 10/2007–09/2022	NCT00480025
Four doses of MAGE vaccine using MAGE-A3 HPV-16	University of Maryland and NIH	Squamous cell carcinoma of the head and neck	I	Completed, no study results posted	NCT00704041
DERMA MAGE-A3 ASCI/MAGE-A3 recombinant Protein vaccine	GlaxoSmithKline	MAGE-A3 positive resected stage III melanoma (IIIB, IIIC, IIITx) ($n = 1,300$ patients)	III	Ongoing 12/2008–10/2016	NCT00796445
MAGE-A3/HPV16 vaccine	University of Maryland	Squamous cell carcinoma of the head and neck	I	Ongoing	NCT00257738
Peptide vaccination using CA antigens from 3 peptides derived from URLC10, CDCA-1 and KOC1	University of Yamanashi	Esophageal squamous cell carcinoma (local, advanced, recurrent or metastatic) ($n = 60$ patients)	II	Ongoing 04/2010–05/2012	NCT01267578

*HPV 16, human papillomavirus strain 16. Information was obtained from www.clinicaltrials.gov to compile this table. This table is not intended to be exhaustive, it provides some necessary information regarding the use of CT antigens for immunotherapy.

to its unusual immunogenic nature to induce high titer of autologous antisera in patients,⁵⁰ NY-ESO-1 has been actively investigated to serve as a suitable target of immunotherapy in NY-ESO-1 expressing tumors^{63,105} (Table 2). For instance, anti-NY-ESO-1 antibody was detected in the serum of ~10–86% of patients of various cancers, which include thyroid cancers, lung cancers, ovarian cancers, breast cancers, bladder cancers, esophageal cancers and melanomas.⁴⁸ About 35 Phase 1 clinical trials have been or are being conducted with His-tagged recombinant NY-ESO-1 worldwide (including the US, Japan and Germany) using different adjuvants (e.g., saponin-based adjuvant, ISCOMATRIX) and in different cancer types (e.g., melanomas).⁴⁸ Despite detecting strong antibody responses, no favorable clinical response was noted in advanced melanoma patients in response to NY-ESO-1 ISCOMATRIX vaccine.¹⁰⁶ However, a study reported in 2008 has shown that ipilimumab [an anti-CTLA-4 (CTLA-4,

cytotoxic T lymphocyte-associated antigen 4 found on cytotoxic T lymphocytes that plays a critical role in regulating natural immune responses¹⁰⁷) monoclonal antibody (marketed as Yervoy by Bristol-Myers Squibb for advanced metastatic melanomas)] therapy combined with NY-ESO-1 vaccine can synergistically enhance the clinical effects in treating advanced metastatic melanomas, since ipilimumab was found to enhance polyfunctional NY-ESO-1-specific T-cell responses to combat metastatic cancer cells.¹⁰⁸ A recent study using a synthetic peptide based on NY-ESO-1 (ESO₁₅₇₋₁₆₅, residues 157–165) was vaccinated in combination with Incomplete Freund's Adjuvant (Montanide ISA-51) and CpG7909 ODN (deoxycytidyl-deoxyguanosin oligodeoxy nucleotides) to a group of 14 patients with melanoma ($n = 7$), non-small cell lung cancer ($n = 4$), ovarian cancer ($n = 1$), sarcoma ($n = 1$) and breast cancer ($n = 1$) with confirmed metastatic and Stages III/IV malignancies. It was found that the vaccine

is able to induce anti-NY-ESO-1 humoral and T-cell mediated response, and positively correlates with survival.¹⁰⁴ However, in a subsequent clinical study in which advanced esophageal ($n = 8$) and prostate cancer patients ($n = 2$) were vaccinated using a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) to efficiently induce NY-ESO-1 antibody, which in turn activated CD4 and CD8 T cell responses, and associated with strong heteroclitic serological responses against 11 tumor antigens (including MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2, XAGE1B and p53). However, the tumor size did not alter, and most subjects in the study eventually died, rendering the vaccine to be ineffective.⁷⁵ Interestingly, no functional study was found in the literature to explore the physiological role of NY-ESO-1 in spermatogenesis regarding its unusual high expression in spermatogonia and primary spermatocytes (Fig. 2) even though its expression in the testis was reported ~15 years ago.⁵⁰

Melanoma-associated antigen (MAGE) gene family. Since the identification of the first MAGE gene in a human melanoma called *MAGE-A1* in 1991,¹⁰⁹ the MAGE family has expanded to include over 60 genes.^{110,111} MAGE genes, as the name implies, are highly expressed in melanoma in particular metastatic melanoma and by germ cells in the testis.^{111,112} All members of MAGE genes share a conserved stretch of sequence of about 200 amino acids known as the MAGE homolog domain (MHD), usually located close to the C-terminus.¹¹¹ Initial studies reported that MAGE genes (e.g., *MAGE-A1*) were silenced in normal adult tissues except male germ cells and, for some of them in the placenta. They have been targets for immunotherapy as they were highly expressed in different types of tumors (e.g., MAGE-A3).⁶³ However, similar to SP17, a few members of the MAGE family were subsequently found to be expressed in normal cells.^{52,113} Interestingly, MAGE-like genes are found in non-mammalian species, including *Drosophila melanogaster* (Fruit fly) and *Danio rerio* (Zebrafish), but not in *Caenorhabditis elegans* (Roundworm), *Saccharomyces cerevisiae* (Baker's yeast) or *Schizosaccharomyces pombe* (Fission yeast), indicating that MAGE genes might have evolutionarily conserved functions and recent studies have shown that MAGE sequences are also found in some flowering plants, such as *Arabidopsis thaliana* (Mouse-ear cress).¹¹⁰

The MAGE family is now composed of two subfamilies MAGE-I and MAGE-II.¹¹¹ MAGE-I family consists of chromosome X-clustered genes including MAGE-A, MAGE-B and MAGE-C groups.¹¹¹ Most MAGE-I family members are CT antigens, rarely expressed in normal adult tissues except germ cells in the testis, but highly expressed in different types of cancer.^{52,59} MAGE-I proteins are crucial to tumorigenesis and cancer cell survival. For instance, MAGE-A3 was found to bind to procaspase-12 to block its activation to caspase-12. This disrupts the caspase-12-mediated cell apoptosis in response to drug-induced insults, resulting in enhanced survival of cancer cells as the result of increased expression of MAGE-A3.¹¹⁴ Recent studies have also shown that several MAGE proteins interact with RING (Really Interesting New Gene, a protein structural domain of zinc finger

type containing a Cys₃HisCys₄ amino acid motif that binds to two zinc cations which plays a key role in protein ubiquitination pathway and cell proliferation^{115,116}) domain-containing proteins (e.g., E3 ubiquitin ligases) and can modify and adjust E3 ubiquitin ligase activity.¹¹⁷ Since MAGE-A antigens are not expressed in normal tissues except germ cells in the testis, they are the targets of immunotherapy. Based on the promising findings using a MAGE-A3-based vaccine from a randomized Phase II study in patients with metastatic melanoma and a double-blinded placebo-controlled Phase II study in patients with resected lung cancer, two Phase III clinical trials have been initiated in patients with melanoma skin cancer and non-small cell lung cancer, which are known as DERMA (Adjuvant Immunotherapy with MAGE-A3 in melanoma) and MAGRIT (MAGE-A3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy), respectively (Table 2).^{111,118,119} In these MAGE-A3 Antigen-Specific Cancer Immunotherapeutic (ASCI) trials, MAGE-A3 recombinant protein and a potent immunostimulant AS15 were used to formulate a vaccine and the results of these two Phase 3 trials will be known by the end of this decade.

On the other hand, members of MAGE-II family (e.g., MAGE-D proteins) are expressed in normal tissues and they are not related to cancer even though some of them are also located on X chromosome. Interestingly, each member also contains a MDH domain (MAGE Homology Domain, a signature domain for MAGE proteins) but the MDH domain is located more distant from the N-terminus.¹¹¹ MAGE-D1 gene, unlike members of MAGE-I family, is downregulated in tumor cells. In addition, protein of MAGE-D1 gene, Maged1, was found to play a role in anti-tumorigenesis in different cell types (and it was also shown to regulate circadian clock functions via its extensive network of interacting partner proteins).¹²⁰ Maged1 is also a binding partner of 75 kD low-affinity neurotrophin receptor (p75^{NTR}), the apoptosis inhibitory protein XIAP, and Dix/MSX homeodomain protein, involving in blocking cell cycle progression and enhancing apoptosis.¹²¹ Necdin, another MAGE-II family member, is a neuron-specific growth suppressor and binds to a cellular transcription factor E2F-1 (E2 promoter binding factor-1), which regulates cell cycle-specific genes.^{122,123} Necdin is known to interact with neurotrophic tyrosine kinase receptor type 1 (TrkA) and p75^{NTR}, and its deletion on the chromosome 15 is associated with Prader-Willi syndrome (PWS, a neurobehavioral disorder and the genetic cause of life-threatening obesity in children).^{124,125} Necdin, similar to Maged1, is also downregulated in primary tumors suggesting a tumor suppressor role, which involves in hematopoietic stem cell quiescence and transcriptional repression.¹²⁴ In short, these findings illustrate that while MAGE proteins are CT antigens, members of the MAGE-II family are having anti-tumor properties.

SSX (synovial sarcoma X). Human synovial sarcoma is an aggressive malignant soft tissue tumor with high recurrence and metastasis as the result of chromosomal translocation. It is characterized by its unique (X;18)(p11.2;q11.2) chromosomal translocation leading to the formation of the SS18-SSX fusion gene that causes tumorigenesis. In synovial sarcoma, the breakpoint-associated genes are fusion genes of SYT from chromosome 18

(also known as SS18), and SSX from the X chromosome.^{126,127} The resulting fusion protein product of SS18 (or SYT) and SSX genes encode nuclear proteins that exhibit opposite transcriptional regulatory activities, with the SS18 protein functions as a transcriptional activator and the SSX proteins as transcriptional repressors.^{127,128} It was shown that the downstream target of the SS18-SSX fusion protein is COM1, a regulator of cell proliferation, in which SS18-SSX downregulated COM1 in synovial sarcoma tissues and cell lines.¹²⁹ The SSX breakpoint multi-gene family is composed of at least 9 genes of SSX1-9, and all are located on chromosome X.¹³⁰ Among the 9 SSX genes, the mRNA transcripts of SSX1, 2, 3, 4, 5 and 7 (but not 6, 8 and 9) are expressed in normal human testes but not in other human tissues.¹³⁰ Among tumor tissues, SSX 1, 2 and 4 are frequently expressed while SSX 3, 5 and 6 are rarely expressed. For SSX 7, 8 and 9, they have yet to be detected.¹³⁰ Similar to other CT antigens, aberrant expression of SSX proteins during malignancy can elicit the production of antibodies. Anti-SSX1, 2, 3 and 4 antibodies have been demonstrated in sera from cancer patients with different tumors including melanoma, colon, breast and ovarian cancers.¹³¹⁻¹³³ Thus, SSX proteins are potential targets of immunotherapy.^{134,135} However, no clinical trials have been conducted using SSX proteins as vaccines. By using a mouse monoclonal antibody that was found to cross-react with SSX2, 3 and 4 (but not SSX1), SSX proteins were found to be localized to the nucleus of spermatogonia and early spermatocytes (e.g., preleptotene, leptotene and zygotene) but not pachytene spermatocytes, round spermatids, elongating/elongated spermatids and spermatozoa in human testes.¹³⁶ This finding thus illustrated the possible involvement of SSX proteins in cell proliferation (e.g., self-renewal of spermatogonia stem cells and spermatogonia) and cell cycle progression. This localization pattern is also consistent with an earlier report which showed that the SS18-SSX fusion protein regulates cell proliferation via its effects on cell proliferation regulator COM1.¹²⁹

Synaptonemal complex protein 1 (SCP-1). SCP-1, also known as HOM-TES-14 (Table 1), is selectively expressed during meiotic prophase of spermatocytes (from zygotene to diplotene)¹³⁷ in human testis at the synaptonemal complexes because it is an integrated component of the synaptonemal complexes.¹³⁷ It is involved in the pairing and recombination of homologous chromosomes during meiosis I.^{137,138} Unlike other CT antigens which are located on chromosome X, SCP-1 is localized on chromosome 1.¹³⁸ SCP-1 is also expressed in neoplastic tissues and tumor cell lines, including malignant gliomas, breast, renal cell and epithelial ovarian cancer.^{138,139} Its elevated expression in epithelial ovarian cancer (EOC) is associated with poor prognosis and reduced survival in EOC patients, and as such, it is being considered a target of immunotherapy.¹⁴⁰ Its restricted expression in pre-meiotic spermatocytes for chromosomal pairing illustrates its significance in meiosis. However, in cancer cells, SCP-1 displayed a cell cycle phase-independent nuclear expression in cancer cells.¹³⁸ Future studies should include studies to examine the role of SCP-1 in mitosis in tumor cells.

Piwil2. Piwi (P-element induced wimpy testis was first identified in *Drosophila*^{141,142}) is a class of genes encoding regulatory

proteins responsible for maintaining differentiation and self-renewal in stem cells, RNA silencing and translational regulation.¹⁴²⁻¹⁴⁵ They are members of the Argonaute family which are highly conserved among species¹⁴⁶ and are the catalytic components of RISC (RNA-induced silencing complex) which is responsible for RNAi to silence gene in normal eukaryotic cells to maintain cell homeostasis.¹⁴⁷ Argonaute proteins bind small non-coding RNAs such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs).^{145,148-151} Miwi is the murine homolog of Piwi (and Hili is the human homolog of Piwi) and it was found to encode a cytoplasmic protein specifically expressed in spermatocytes and spermatids that is essential for spermatogenesis, since *Miwi*^{-/-} mice displayed spermatogenic arrest at round spermatid, disrupting spermiogenesis.¹⁵² Among the genes of the Piwi family, they all contain the conserved PAZ domain (a ~110-amino acid stretch of sequence found in Piwi, Argo and Zwiille/Pinhead proteins in *C. elegans*, *Drosophila* and humans) and Piwi domain (Piwi is a protein domain homologous to piwi proteins that bind and cleave RNA).¹⁵³ A stem-cell protein Piwil2 (Piwi-like 2) was found to be specifically expressed in the testis of humans and mice, restricted to spermatogonia and early spermatocytes, with enhanced expression in human testicular seminomas (but not in testicular non-seminomatous tumors) and also in different tumors, including prostate, breast, gastrointestinal, ovarian and endometrial cancer of humans.¹⁵⁴ As such, Piwil2 is a CT antigen and an oncogene.⁶⁰ Piwil2 exerts its effects by inhibiting cell apoptosis and promoting cell proliferation via the Stat3/Bcl-X_L signaling pathway.¹⁵⁴ A recent study has shown that Piwil2 regulates chromatin modifications such as chromatin condensation that affects DNA repair and its overexpression in cancer cells could lead to cellular cisplatin resistance.¹⁵⁵ Several Piwil2-like (PL2L) proteins have recently been found in various human tumors that promote tumor cell survival and proliferation via the Stat3/Bcl2 pathway.¹⁵⁶ Thus, by blocking the expression of this gene during tumorigenesis would reduce the uncontrolled expansion of malignant cells and since it is a CT antigen, it can become a target of immunotherapy and/or diagnostic/prognosis biomarker.

ACRBP (acrosin binding protein). ACRBP, also known as OY-TES-1 or CT13 (cancer/testis antigen 23), is an acrosin binding protein located on chromosome 12 (Table 1).¹⁵⁷ OY-TES-1 is the human homolog of proacrosin binding protein sp32 precursor first found in pig and mouse testis.¹⁵⁸ Besides highly expressed in the testis and localized to the mature spermatozoa at the acrosomal region and not other somatic cells (Fig. 2), it is expressed at high levels in malignant tissues including bladder, breast, lung, liver, colon and ovarian cancers.^{157,159,160} Anti-OY-TES-1 antibody was detected in ~10% of the ovarian cancer patients whose tumors expressed this antigen.¹⁶⁰ Its elevated expression in ovarian cancers was found to associate with reduced survival time and faster relapse in ovarian cancer patients,¹⁵⁹ illustrating OY-TES-1 can be a target of immunotherapy. A recent study has shown that the mitotic spindle protein NuMA is the binding partner of this acrosin binding protein, and as such, OY-TES-1 affects mitotic spindle assembly during tumor cell proliferation.¹⁵⁹

CT antigen expression during spermatogenesis. The role of CT antigens in spermatogenesis remain largely obscure (with the unusual exception of SCP-1 which is known to be involved in synaptonemal complex formation) since virtually no functional studies are found in the literature to explore their roles in spermatogenesis except for several studies that examine their immunohistochemical localization in human testes (or normal rodent testes) versus patients' samples having different cancers. These findings are briefly summarized in **Figure 2** and discussed herein. However, based on these limited findings, some obvious experiments can be conducted in future studies to understand the role of these transiently expressed cancer genes during spermatogenesis, in particular stem cell proliferation, cell cycle progression, meiosis and spermiogenesis. These studies will assist the understanding on the involvement of CT antigens in these cellular events but also enhance our understanding of the molecular mechanism(s) underlying tumorigenesis and metastasis when these CA antigens are highly expressed and/or activated.

A mouse monoclonal antibody, designated 57B, that was developed against recombinant MAGE-3 protein (i.e., an anti-MAGE-3 antibody, but it was found to cross-react also with MAGE-1, -4, -6 and -12,⁵² which are members of the MAGE-A group¹⁶¹ in MAGE-I subfamily) has been used for immunohistochemistry analysis to examine the association of MAGE-3 with specific germ cell types.⁵² MAGE-A was found to be highly expressed in spermatocytes, early spermatocytes (e.g., leptotene, zygotene and diplotene spermatocytes), but somewhat diminished in pachytene spermatocytes and round spermatids, and appeared to be localized more to the nucleus than the cytoplasm, but not expressed in elongating spermatids and spermatozoa, nor in Sertoli and Leydig cells⁵² (**Fig. 2**). Interestingly, this pattern of staining was almost identical in a subsequent study using a specific anti-MAGE-1 monoclonal antibody designated MA454 except that the localization of MAGE-1 is more prominent in the cytosol and at the perinuclear region of spermatogonia, spermatocytes and round spermatids.¹⁶² Collectively, these findings^{52,162} show that MAGE-3, -4, -6 and -12 may have slightly different cellular distribution, which are nuclear bound, versus MAGE-1 which was localized more to the cytoplasm. Furthermore, MAGE proteins may be intimately related to cell cycle progression, to prepare germ cells for meiosis I and II, and early phase of spermiogenesis but not involved in spermiation.

Magphins, a new group of MAGE proteins composed of three isoforms of magphinin- α , - β and - γ , are found in the mouse testis and ovary, restricted to the male and female germ cells, regulating cell proliferation during gametogenesis.¹⁶³ However, magphinin- α and - β , unlike other CT antigens, are also detected in the epididymis and brain.¹⁶³ Overexpression of magphinin- α in GC1spg cells (GC1spg cell line was established by immortalizing spermatogenic cells with SV40 antigen¹⁶⁴) or overexpression of magphinin- α , - β or - γ in Saos-2 cells (a non-transformed cell line derived from primary osteosarcoma¹⁶⁵) were found to inhibit cell proliferation by promoting cell cycle arrest,¹⁶³ illustrating their involvement in cell cycle progression, and perhaps SSC/spermatogonia proliferation for

their self-renewal to maintain the proper SSC population at the stem cell niche. When the localization of magphilins in the seminiferous epithelium of adult mouse testis were examined, magphinin- β was found to be abundantly expressed by spermatogonia and spermatocytes in the cytosol. On the other hand, both cytosol and nucleus were stained with magphinin- β in secondary spermatocytes and round spermatids, which persisted through elongated spermatids and spermatozoa at spermiation¹⁶³ (see **Fig. 2**). Studies by immunohistochemistry have shown that NY-ESO-1 was prominently expressed by spermatogonia, early spermatocytes through pachytene spermatocytes but not in any post-meiotic spermatogenic cells including spermatids and spermatozoa in human testis, and also not in Sertoli and Leydig cells^{101,102} (**Fig. 2**). Furthermore, this pattern of expression is somewhat similar to the expression of SCP-1¹³⁷ and Piwil2¹⁵⁴ based on in situ hybridization (and immunogold electron microscopy) and immunohistochemistry analysis, respectively. For SCP-1, it is a structural component of synaptonemal complexes involving in pairing of the homologous chromosomes in zygote through diplotene spermatocytes and it is restricted mostly to these spermatocytes,¹³⁷ whereas Piwil2 is involved in spermatogonia and SSC renewal and differentiation and is therefore restricted to these cells¹⁵⁴ in the human testis.

However, several other CT antigens are restrictively expressed and localized to spermatozoa in the testis such as SP17, and OY-TES-1 is restricted to elongating/elongated spermatids and spermatozoa instead of the less advanced germ cell types (**Fig. 2**). These findings regarding the expression and localization of several CT antigens in germ cells during spermatogenesis clearly illustrate that much work is needed to delineate the function of these proteins in spermatogenesis. These findings, if available, should provide insightful information regarding their role and their aberrant expression during tumorigenesis.

Concluding Remarks and Future Perspectives

As summarized above, while the physiological function for virtually all the CT antigens during spermatogenesis remain unknown, several of these CT antigens are emerging as proteins intimately related to: (1) spermatogonial stem cell/spermatogonial self-renewal via mitosis (e.g., Piwil2), (2) transcription regulation (e.g., SS18-SSX proteins), (3) chromosomal pairing in spermatocytes to prepare for meiosis I (e.g., SCP-1), (4) germ cell apoptosis (e.g., MAGE proteins. Piwil2), (5) spermiogenesis (e.g., OY-TES-1) and (6) sperm motility (e.g., SP17). In short, this information, as summarized in **Figure 2**, thus illustrates that CT antigens are involved in virtually all the different phases of cellular events pertinent to spermatogenesis. With the recent advances in molecular and cell biology, and biochemical and molecular techniques to manipulate the expression of different genes and/or proteins such as by RNAi utilizing siRNA duplexes, shRNA, and/or lentiviruses/adenoviruses, we will gain a lot of insightful information regarding the molecular mechanism(s) by which these CT antigens regulate spermatogenesis. These findings should be helpful to design better chemotherapeutic drugs to treat different tumors in humans.

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