Molecular cloning, gene expression analysis and characterization of spermatogenesis related genes

精子形成関連遺伝子のクローニング、発現解析ならびに機能分析

Doctoral Thesis

For

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Abbreviations

[α-^{32}P] dCTP: [α-^{32}P] 2'-deoxycitidine 5'-triphosphate

AQP: Aquaporin

ATP: adenosine triphosphate

bHLH: basic helix-loop-helix

cAMP: cyclic adenosine 3′,5′- monophosphate

CCCAP, centrosomal colon cancer autoantigen protein

cDNA: complementary DNA

CFTR: cystic fibrosis transmembrane regulator

ChiP: chromatin immunoprecipitation

CREB: cAMP responsive element binding protein

CT: cancer testis antigen

DAB: diaminobenzidine

DD: differential display

DDBJ: DNA data bank of Japan

DES: diethylstilbestrol

DIG: digoxigenin

EST: expressed sequence tags

FSH: follicle stimulating hormone

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HPG: Hypothalamus-Pituitary-Gonads

HRP: horseradish peroxidase

IHC: immunohistochemistry

ISH: *in situ* hybridization

LH: luteinizing hormone

MAPK: mitogen-activated protein kinase

miRNA: microRNA

MOPS: 3-(N-morpholino) propanesulfonic acid

NCBI: National Center for Biotechnology Information

NRP: nucleolin related protein

ORF: open reading frame

PCR: polymerase chain reaction

pi RNAs: Piwi-interacting RNAs

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RACE: rapid amplification of cDNA ends

RGN: regucalcin

RT-PCR: reverse transcription polymerase chain reaction

SCL: stem-cell leukemia

SD rats: Sprague-Dawley rats

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEREX: serological analysis of recombinant cDNA expression

si RNAs: short interfering RNAs

SRF1: spermatogenesis related factor 1

SSC: saline sodium citrate

SSH: suppression subtractive hybridization

TAE: Tris-Acetate-EDTA

Tal-1: T cell acute-lymphocytic leukemia

TCDD: 2,3,7,8- tetrachlorodibenzodioxin

TSP: testis-specific protease

TUSC: tumor suppressor candidate

TZF: testicular zinc finger protein

CHAPTER I

General Introduction

1.1 Sexual reproduction

Sexual reproduction is an important biological process; it takes place in majority of animals which mixes the genomes of parents to produce offspring. The produced individuals differ genetically from their parents and even within them [1]. The sexual reproduction is well organized process occurs in diploid organisms which include two sets of chromosomes in their cells inherited from each parent. The haploid cells which contain only one set of chromosomes commonly are specialized for sexual reproduction, which fuse with another haploid cell to mix their genomes and to maintain the diploid state [2].

Mitosis, meiosis, and fertilization

Continuation of sexual maturation process requires unique type of cell division which called meiosis. In meiosis, diploid cells divide to haploid progeny cells, which develop into highly specialized gametes [3]. In animals, male produces haploid sperms whereas female produces haploid eggs, which fuse at fertilization to produce diploid cell containing new combination of chromosomes. Moreover, mitosis is essential to form multicellular organism and proliferate diploid cells [4]. The haploid gametes exists only briefly and do not divide, which specialize for sexual fusion. Briefly, during meiosis the duplicated maternal and paternal homolog pairs up alongside each other and exchange genetic information through the process of genetic recombination [5]. Furthermore, they line up and segregate into daughter cells. The haploid gametes and their specialized diploid precursors are called germ cells, while somatic cells are the cells which form the rest of the body. Somatic cells are necessary for germ cells to survive and develop, whereas germ cells only transmit their DNA to next generations [2].

In the sexually reproductive animals there are two major different types of gametes which are sperm and egg. Each sperm or egg is optimized for the propagation of the genes they carry. The egg which aids the survival of the maternal genes is nonmotile and larger than sperms. In contrast, sperm is optimized to propagate the paternal genes; it is highly motile for efficient and successful fertilization [6]. Sperms which are produced continuously in mammalian testes by meiosis, begin when mammalian reach puberty. Immature germ cells, called spermatogonia, are located around the outer edge of seminiferous tubule which called the basal membrane. Spermatogonia divide by mitosis for limited time, stop proliferation and start meiosis to become primary spermatocytes and then secondary spermatocytes. Secondary spermatocytes differentiate into spermsatids and continue to sperm after meiosis [7].

Sexual reproduction and genetic diversity

Through sexual reproduction, animals inherit two full sets of chromosomes, one from each parent. Each set contains autosome and sex chromosome, the former is common between species, while sex chromosome differ according to the sex type. During meiosis, it is important to communicate between homologous partner chromosomes to segregate into different daughter cells [8]. The genetic differences happen due to the randomly distribution of the maternal and paternal chromosomes to haploid cells. Another reason for genetic differences is the production of novel hybrid versions of each chromosome by crossing over, even the similarity of paternal and maternal chromosomes [9]. Therefore, there are several advantages for sexual reproduction. The individuals produced have unique genetic combinations due to different reshuffled genes, which help species to survive in variable types of environment. Another benefit is the elimination of the deleterious genes from the populations [1].

1.2 Spermatogenesis

Spermatogenesis is a highly sophisticated process involved in the transmission of genetic heritage. Spermatogenesis occurs in successive mitotic, meiotic and post-meiotic phases in the testis, and many genes expressed systematically. The testis is a complex organ playing as a host of one of the most complicated mass cell divisions occurring in postnatal life (it is the initial host of morphological, biochemical and physiological processes which is necessary for testicular maturation) [10,11]. In mammalian testis, spermatogenesis takes place in the seminiferous tubule, which is the functional unit that produces haploid spermatozoa from diploid spermatogonia [10]. Sertoli cells play an important role in the transfer of spermatocytes to the adluiminal compartment through blood testis barriers during transit of spermatocytes [12]. Recent studies mentioned that rat Sertoli cells help the mechanical attachment of spermatid heads during elongation and maturation [13].

Several reports demonstrated different reasons for abnormal spermatogenesis process which result in human infertility and azoospermia. The male infertility in rats, mice and human by the appropriate classification of impaired spermatogenesis, was investigated. The numbers of Sertoli cells and spermatogonia including stem cells were significantly reduced in azoospermic patients with defined spermatogenic defects [14].

In the seminiferous tubules, spermatogonia stem cells attach to the basal membrane, divide by mitosis, and undergo meiosis producing spermatids from spermatocytes (Fig.1-1). This is known as the cycle of the seminiferous epithelium [15, 16].

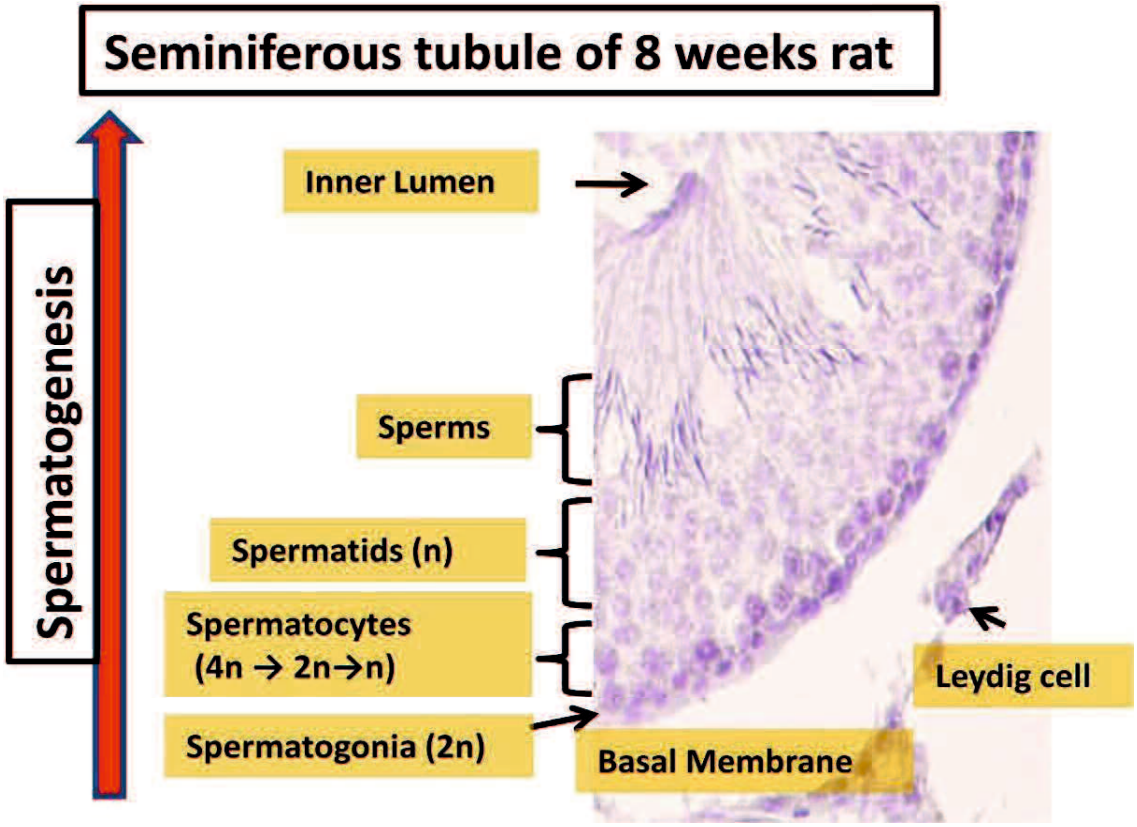

Fig.1-1. HE staining showing seminiferous tubule of mature 8 weeks old male rat

Besides germ cells, the seminiferous epithelium is composed of somatic Sertoli cells which support spermatogenesis. Sertoli cells play an important role in spermatogenesis by several ways: (1) providing suitable environment necessary for germ cells development, (2) supporting cell polarity in cell-cell interactions, and (3) creating an immunological barrier from the systemic circulation [17-19]. The spermatogenesis is also stimulated by Leydig cells in the interstitium which produce testosterone to support Sertoli and germ cell function and to regulate germ cell maturation [20].

Spermatogenesis is highly complex process, which is precisely regulated by the endocrine system; Hypothalamus-Pituitary-Gonads (HPG) axis [21-25]. Pituitary follicle stimulating

hormone (FSH) is one of the key players to regulate spermatogenesis [21, 24]. Testosterone production from interstitial Leydig cells stimulated by Luteinizing hormone (LH) is essential for the process of spermatogenesis and to maintain Sertoli cells function, leading to perform germ cell maturation [21-23]. Estrogen is produced by the interstitial Leydig cells, Sertoli cells, and germ cells in the seminiferous epithelium, and it is also crucial for germ cell development [21, 25]. These hormonal regulations are important for proper spermatogenesis. To clarify the effect of FSH, LH, inhibin and testosterone secretions on sperm motility, short period vasectomy of male rats was performed, reduction of sperm motility, and degenerative spermatids in the epididymides of vasectomized rats were observed [26].

Stimulation of Sertoli cells by FSH activates the cAMP responsive element binding protein (CREB), which is necessary for the regulation of spermatogenesis [27]. Mutations of the cystic fibrosis transmembrane conductance regulator (CFTR), which cause infertility in Caucasians, were studied. The study revealed the essential role of CFTR in regulating the cAMP-CREB signaling pathway in Sertoli cells, while defect of which resulted in impaired spermatogenesis and azoospermia [28].

Endocrine disrupting chemicals have adverse effects on reproduction, and the study of their effect in natural hormones leads to the understanding of natural sexual hormone signaling [29]. Furthermore, sterility and abnormal spermatogenesis in offspring due to the exposure of endocrine disruptor's effect on their parents was also reported. Maternal exposure to low dose of diethylstilbestrol (DES), which reduced the expression of testicular endocrine function in male rat offspring, reduced the expression of the luteinizing hormone receptor (LHr) but induced high level of AR mRNA expression to recover the low levels of plasma testosterone [30].

1.3 Gene expression in individual stages of spermatogenesis

Spermatogenesis is a complex, programmed process that involves the ordered expression of many genes within the testes. Study of gene expression regulation during spermatogenesis is most important to understand this process. These gene products expressed during this process encode proteins necessary for different phases of germ cell development. Analysis of these gene products may lead to the understanding of reproduction and certain diseases because they mirror total gene expression on our body. Specific roles of several genes in spermatogenic cells have been clarified. It is important to study many factors affect on the gene expression according to transcriptional, translational and post-translational regulation [31].

The DNA microarray was performed for screening spermatogenesis related genes in rats and mice using isolated spermatogenic cell-derived mRNA as probes after separation by flow cytometry (Table1-1) [35]. As an example, using affymetrix gene chips represented expected genes which have different expression profile between infants and mature mice [32, 33]. The expression of these genes was confirmed by quantitative RT-PCR (qRT-PCR) and Northern blotting [34]. Even though, the expected functions of some of these gene products have been elucidated by comparing the DNA sequence database, functions of many genes remain unknown [36].

Table 1-1. Appearance of specific spermatogenic cells in the developing testis according to rat and mouse of the postnatal days. (*Malkov et al.*1998)

Stage no.	Description of developmental stage	Age in rat	Age in mouse
I	Spermatogonia and somatic cells only	Days 6–7	Days 6–7
II	Initiation of meiosis I—leptotene cells	Days 13–14	Day 10
III	Appearance of zygotene cells	Days 17–18	Day 12
IV	Appearance of early pachytene cells	Days 19–20	Day 14
V	Appearance of late pachytene cells	Days 22–23	Days 17–18
VI	Appearance of round spermatids	Days 24–25	Days 20–21
VII	Appearance of elongating spermatids	Days 30–31	Days 24–25
VIII	Appearance of elongated spermatozoa	Days 36–37	Days 27–28

More than 15,000 full-length mammalian cDNA sequences, or expressed sequence tags (EST), have been isolated, and by using this information, spermatogenesis-related genes have been identified and determined. Moreover, full length cDNAs or ESTs were analyzed [37]. Rapid amplification of cDNA ends (RACE) clarified the full length of the individual cDNA information which is necessary to analyze the gene expression [38].

Finally, proteomics study is required to understand the individual protein expression [39]. According to the expected protein information, specific antibodies were constructed. Protein localization in the testis was clarified by immunohistochemistry using antibodies [40].

According to these procedures, spermatogenic stage specific gene expression was determined. Spermatogonia specific expressing gene, GON-SJTU1 product was identified and analyzed in rat testis. Analysis of the gene and its product revealed high expression from day 1 to 15 and then decreased; the main expression was detected in spermatogonia suggesting a role in early

stage in spermatogenesis [41]. Further studies determined the expression of BMP4 growth factor in rat's spermatogonia derived cell lines (GC-6spg). The result also showed KIT expression is observed in GC-6spg cells, this protein is commonly used as early marker for differentiating spermatogonia [42].

The task of spermatogonia is to provide self renewal and differentiation to be able to enter meiosis. Differentiated spermatogonia develop into primary and secondary spermatocytes; this is necessary step to continue spermiogenesis during which the spermatids alter their shapes and contents to produce the typical shape of spermatozoa [43-45]. During these different stages many genes and their products are expressed suggesting the roles in this process. The mRNA and protein expression of MIF4GD (MIF4G domain containing protein), which has function of translational regulator and has three isoforms, was up-regulated in pachytene spermatocytes and haploid spermatids [46].

Finally, expression of several genes in spermatozoa is reported. Expression of Proacrosin, the precursor of acrosin, which is the major proteinase present in the acrosome of mature spermatozoa, was observed. As the expression of Proacrosin gene starts in Pachytene spermatocytes and higher expression in round spermatids was observed, its role in sperm maturation is expected [47]. Aquaporin (AQP), which is essential for water permeability through cellular membrane, was studied. This protein is classified to several families, and AQP7 was found to be expressed during rat testis maturation. AQP7 protein was localized in the spermatids, whereas AQP8 was in spermatocytes; and they may be related to sperm maturation [48]. Moreover, the tandem repeated gene on the mammalian Y chromosome (TSPY) expression was investigated. TSPY initially expressed in elongated spermatids in adult testis. Analysis of

GST pull-down assay clarified the co-localization of histones with this protein, suggesting the role of TSPY in the cytoplasm of elongated spermatids, nessary for sperm maturation [49].

1.4 Expected functions of spermatogenesis related gene products

Expression specificity and gene profiling data during spermatogenic processes in the testes may help to elucidate the biological functions of these gene products, and these can be used as genetic markers to diagnose infertility and also certain diseases. According to the expected function many researchers explored spermatogenesis related genes. Several studies evaluated the different expression profiles in the germ cells between fertile and infertile groups or between infants and mature groups [50, 51]. Moreover, other reports studied the effect of polymorphisms and mutations in spermatogenesis related genes on infertility [52].

Analysis of spermatogenesis related genes revealed several candidates, involved in different and wide range of biological activities. According to the analysis, they could be classified to the following expected functions. Among this group, SRF1(spermatogenesis related factor 1), which is suggested as a molecular motor such as kinesin-related protein, and gene expression was testis specific and started from 5 weeks of rat testis mainly in spermatocytes. The gene product may be deeply related to cell division during meiosis [53]. Expression of SRF2 which codes for RabGAP/TBC protein was studied. Up-regulation of this gene expression during maturation suggested its role in membrane trafficking during the process of meiosis. Moreover, reduction of its expression by 2,3,7,8- tetrachlorodibenzodioxin (TCDD) treatment suggested that it could be a promising endocrine disruptor biomarker [54]. Expression of Hsp20 (alphaB crystalline related protein) or small heat shock protein, which has function of molecular chaperon, was detected from week 3 rat testis while the strongest expression was in week 15. The expression of Hsp20 was mainly observed in spermatocytes and round spermatids [55]. Expression of rat sperm flagellum-movement associated protein gene, necessary for sperm cell motility which supplies

ATP as chemical energy, was also up-regulated during spermatogenesis mainly in the spermatocytes [56].

Moreover, tumor suppressor candidates were analyzed. Expression of Ha-ras suppressor family member 5, which modulate the Ha-ras signaling cascade, was investigated. *Hrasls5* gene product may involve in spermatogenesis beside its functions as tumor suppressor [57]. Tumor antigens commonly expressed in normal testis were also studied. Expression of the rat homolog of the serologically defined colon cancer antigen 8 gene (*Sdccag8*) was rat testis specific [58].

Many vertebrate plasma membrane transporters are reported, and they transport a number of divalent cations across plasma membrane, and TUSC3, MagT1 and NIPA2 are known to be specific for Mg^{2+} transport [59, 60]. Magnesium ion was deeply involved in the significant increase of androgenic enzymes activities in the rat testis [61]. Moreover, analysis of calcium, magnesium, zinc and copper in blood and seminal plasma, showed the essential role of these ions in spermatogenesis and fertility [62]. Several reports have described the crucial role of proper Ca^{2+} homeostasis regulation to spermatogenesis and male fertility. Recent studies focused on regucalcin (RGN), which is Ca^{2+} binding protein and it regulates calcium intracellular homeostasis by modulating the activity of enzymes which particularly control spermatogenesis. This result showed that RGN is expressed in Leydig and Sertoli cells, as well as in all types of germ cells in the testis, prostate, epididymis, and seminal vesicles of rat and human, suggesting the important role of calcium on spermatogenesis [63].

Several splicing variants are deeply involved in spermatogenesis. Smaller alternative transcript of mouse alphaT-catenin truncated isoform which is expressed in mice testes was reported [64]. Regarding other expected function, a nucleolin related protein (*NRP*) gene, a ribosomal RNA

transcription factor which is expressed during rat spermatogenesis, was investigated. The domain structure of rat NRP revealed a novel variant, suggested its importance on spermatogenesis by the transcriptional modification of this variant [65]. The expression of LKB1, which is known as a tumor suppressor and its mutation causes Peutz-Jeghers syndrome was studied. Two splice variants of this gene in mammals were reported which are widely expressed in rodent and human tissues. A short novel splice variant has a crucial role in spermiogenesis and male fertility. Knockout mice lacking this variant were sterile, with reduced number of mature spermatozoa in the epididymis with abnormal heads [66].

Beside the different expected functions of individual gene products, non-coding RNAs are important. Recent reports indicated the expression of non-coding RNAs which are classified under three main headings; microRNA (miRNA), Piwi-interacting RNAs (piRNAs), and short interfering RNAs (siRNAs) during spermatogenesis [67, 68]. FSH and androgens act on Sertoli cells to control the expression of miR-23b that operate to regulate cell adhesion pathways and male fertility [68].

1.5 Gene products related to diseases

Gene and protein analysis concerning spermatogenesis related genes were studied in several reports. These genes and their products are involved in several biological activities, and some of them are deeply related to many kinds of diseases and abnormalities. TSP50 protein coded gene, which is abnormally activated in the breast cancer, was studied. This protein expression was down-regulated in the testicular germ cell tumors in human and rodent [69].

DNA methylation, is deeply involved in the epigetic regulation involved on gene expression especially in germ lines. Epigenetic alteration in sperm DNA associated with testicular cancer chemotherapy revealed altered DNA methylation patterns in spermatozoa and improved the animal survival [70]. Homozygous deletion or methylation of *Tusc3* gene leads to several types of cancer such as, prostate cancer [71-74], ovarian cancer [75-77], gastric cancer [78], osteosarcoma [79], pancreatic adenocarcinoma [80-82], lymph node tumors [83], and breast and pancreatic cancer [84].

Tumor antigens commonly expressed in normal testis are interesting target to analyze. They can be used for cancer diagnosis, and are promising tools for cancer immunotherapy [85-87] and can be used to understand the process of spermatogenesis. Cancer-related gene expressions were up-regulated during sexual maturation and are interesting targets to analyze because of the expected common functions and mechanisms between cancer and spermatogenic stem cell development and differentiation.

Cancer/testis (CT) antigens were also studied, which are protein antigens with normal expression restricted to adult testicular germ cells, and yet are aberrantly activated and expressed in a proportion of various types of human cancer. Several reports addressed the important role of cancer testis antigens, which express normally during spermatogenesis beside the frequent

detection in tumors. To understand the functional relevance of this group to tumorigenesis, several proteins were identified. FMR1NB, NXF2, MAGEA5, FSIP1, and STARD6 are classified as CT antigens, and are required for accurate chromosome segregation in tumor cells. These proteins are promising targets to consider cancer treatment according to an antimitotic mechanism [85]. Colon cancer specific antigens have been screened by a recombinant cDNA expression library (SEREX) from an individual cancer patient, and they are promising candidates as cancer-testis (CT) antigen genes [88]. The rat homolog of the serologically defined colon cancer antigen 8 gene (*Sdccag8*), which was screened by SEREX, and its expression was rat testis specific [58]. The gene product was identified as serologically defined colon cancer antigen 8 (SDCCAG8) and centrosomal colon cancer autoantigen protein (CCCAP), and its C-terminal portion was identical to the serologically defined human colon cancer autoantigen (NY-CO-8) [88, 89].

Recent study showed tumor suppressor gene *Tusc3* is associated with mental retardation. TUSC3 is prostate cancer suppressor and deeply related to mental retardation, especially the non-syndromic type [90-95]. Linkage analysis indicated a novel nonsense mutation in the second exon of *Tusc3* gene leading to mental retardation [90]. Moreover, some disorders reported due to deficiency of magnesium ion. The importance of magnesium during spermatogenesis is associated with the enzyme activation, and a reduction of magnesium may cause prostatitis and other disorders [96].

1.6 Thesis objectives and contents

The aim of this study is to screen the spermatogenesis related genes, analyze their expression, and classify these genes according to the expected functions as the first step. There are several genes, commonly express during spermatogenesis in somatic cells, and some of them are related to diseases such as cancer and tumor suppression. In this study, I have screened genes specifically expressed in developing testis, characterized the expression, and classified them according to the expected functions. Data deposition, classification and analysis of these genes and proteins in developing testis are important to clarify the normal spermatogenesis and also abnormal process, such as tumorigenesis and male infertility. This knowledge may open the door to diagnose tumors, male infertility and also to elucidate the mechanism of the effect of endocrine disruptors on spermatogenesis. In the present study, I chose some candidate genes which are up-regulated during the sexual maturation. While the functions of some of these have been determined by comparing DNA sequences in the database, many functions remain unknown.

In Chapter I, I have summarized the background of the study. In chapter II, I focused on one candidate gene *LOC290876*, of unknown biological function. I analyzed its expression in maturing testes, and elucidated its expected function in spermatogenesis. Gene expression was testis specific, increased at week 7, and continued for week 15. I identified two splice variants of *LOC290876.*

In chapter III, a novel CT antigen candidate was analyzed. I studied the expression of the T-cell acute-lymphocytic leukemia (*Tal-1*) or the stem-cell leukemia (SCL) gene in maturing rat testes which is expected to be as promising tool for diagnosing T-ALL. Gene expression was

testis specific, increased at week 7, and continued for week 15. I clarified the rat 5' end sequence according to the gene database and predicted protein size. According to the known function of *Tal-1*, which has functions in angiogenesis and hematopoietic differentiation, erythropoiesis in bone marrow during embryogenesis, as well as T-ALL development; I expected additional function on spermatogenesis.

In chapter IV, I analyzed the expression of *Tusc3*, a tumor suppressor candidate gene 3, during spermatogenesis. The expression was up-regulated in developing rat testis. Gene expression was detected in the testis, increased at week 3, and continued for week 15. *Tusc3* gene expression was analyzed in several types of cancer, and its involvement in several biological functions such as magnesium transporter, expected role in protein N-glycosylation, and relation to tumor suppression and mental retardation. *Tusc3* expression may deeply involve in spermatogenesis.

Chapter II

Expression of a Novel Sphingosine 1-Phosphate Receptor Motif Protein Gene in Maturing Rat Testes

2.1 Abstract

A novel sphingosine 1-phosphate receptor type 5 motif-containing gene, *LOC290876*, have been screened from maturing rat testes by differential display. Gene expression was testis-specific, increased at week 7, and continued for 15 weeks. PCR analysis clarified two gene transcript isoforms, which were expressed at the same level in all samples detected in Northern blot. The deduced amino acid sequences of the two isoforms revealed differences in carboxyl terminal sequences. Gene and protein expression in the testes was dominant in the spermatocytes, and protein expression was localized to the nucleus. Taken together, these findings suggest that the *LOC290876*-encoded gene product is not involved in sphingosine signaling, but has distinct roles in the nucleus during the processes of spermatocyte maturation and meiosis producing spermatids.

2.2 Introduction

Spermatogenesis is a complex, programmed process that involves the ordered expression of many genes within the testes. In the seminiferous tubules, spermatogonia stem cells attach to the basal membrane, divide by mitosis, and undergo meiosis producing spermatids from spermatocytes. This is known as the cycle of the seminiferous epithelium [15, 16]. More than 15,000 full-length mammalian cDNA sequences, or expressed sequence tags (EST), have been isolated, [38] and using this information, spermatogenesis-related genes have been identified and determined.

The DNA microarray is a powerful tool for screening these genes using isolated spermatogenic cell-derived mRNA as probes after separation by flow cytometry [32-35]. Using Affymetrix gene chips, wide genome analysis based on gene expression difference was carried out between 7-day- old and pachytene spermatocytes from 18-day old mice, which confirmed the pre-meiotic and mitotic expression for several genes [32]. Analysis of cell-specific patterns revealed several transcript groups with different biological and chronological activities of testicular cell types during the spermatogenesis progress [33]. Pang *et al.* used DNA microarray as tool for different stages of germ cell development, specific functions of the encoded products which differentially expressed in spermatogenesis were suggested [34]. Using flow cytometry analysis for rats comparing to mice based on spermatogenesis developmental schedule, Malkov *et al.* suggested detailed stages. Somatic cells and spermatogonia were observed at 6-7 days of postnatal days of rat testis, 13-14 days for leptotene, 17-18 for zygotene 19-23 for pachytene spermatocytes, respectively [35].

Based on DNA microarray technique, many spermatogenesis candidate genes have been isolated and analyzed. While the functions of some of these have been determined by comparing DNA sequences in the database, many functions remain unknown [36]. Understanding expression specificity and gene profiling during spermatogenic processes in the testes should help to elucidate the biological functions of these gene products, which can be used as genetic markers to diagnose human infertility [50, 52]. Montjean *et al*. compared the gene expression profile in spermatozoa of infertile and fertile men by microarray analysis, reduction up to 33 folds of the gene expression and sperm motility was observed [50]. Nishinmune and Tanaka reported mutations and polymorphisms as factors causing infertility [52].

Recently, Yang *et al.* described the expression of novel gene *GON-SJTU1* in rat testes, which was detected by 15 postnatal days, and then dramatically decreased after sexual maturation [41]. The expression of this gene product was examined by Northern and Western blotting; furthermore, *In situ* hybridization and Immunohistochemistry were applied to determine their localization. Although the function of this gene is not well understood, information on the gene should be useful in diagnosing diseases such as cancer and infertility, as well as in understanding the molecular mechanism of spermatogenesis.

Several specific genes have been screened from maturing rat testes by differential display (DD), identified, and characterized. 3-weeks and 7 weeks-old rat testes representing infant and adult rats were used in DD, respectively. In the present study, I focused on candidate gene *LOC290876*, of unknown biological function and analyzed its expression in maturing testes, which exhibited increased expression during maturation and elucidated its expected function in spermatogenesis.

2.3 Materials and Methods

Animals and samples treatment

Sprague-Dawley (SD) rats were purchased from CLEA Japan (Tokyo). All rats were sacrificed by ether inhalation; organs were quickly dissected, removed and kept in -80 °C for further investigation. Total RNA from testes at various developmental stages and from individual organs of 9-week-old male and female rats was prepared using TRIzol reagent (Life Technologies, Carlsbad, CA), following the manufacturer's recommendations. DD was performed using 3-week- and 7-week-old rat testis RNA as template. Candidate cDNAs, showing higher expression at week 7 than at week 3, were cloned and sequenced, and their nucleotide sequences were compared with those in the National Center for Biotechnology Information (NCBI) DNA database. I focused on one candidate, sphingosine 1-phosphate receptor motif protein gene *LOC290876*, accession no.NM_001037182, with unknown function. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

RT-PCR

Gene expression was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Northern blotting. RT-PCR primer sequences of *LOC290876* were designed as follows: upstream primer 5'-CCCATGACACAGGGTACTTGTCTG-3', located in exon 6, and downstream primer 5'-GAACCAGTTCTGAGGTGACCTTGT-3', located in exon 9. cDNA was synthesized from 1 μg of RNA using a first-strand cDNA synthesis kit, including Super Script III Reverse Transcriptase (Life Technologies). PCR was done using TaKaRa Ex. Taq DNA polymerase (Takara Bio, Shiga, Japan), according to the following schedule: 94°C for

5 min., and then 94°C for 30 s, 60°C for 30 s, 72°C for 1 min., for 30 cycles, and finally 72°C for 10 min. The expected amplification size according to the deposited sequence was 489 bp. Mammalian *Gapdh* primers 5'- AGTCGGAGTGAACGGATTTGG -3', and antisense primer 5'-AGTTGTCATGGATGACCTTGG -3' were used for PCR amplification as internal control. Electrophoresis was done using 1.5 % Agarose gel in 1 X TAE buffer.

Northern blotting

For Northern blotting, 5 µg of total SD rat RNA was electrophoresed on a formaldehyde-containing 1% agarose gel in 1X MOPS after incubation at 65 °C for 15 min. for the RNA samples. After electrophoresis, the membranes were stained using ethidium bromide for 20 min. shaking and then washed using distilled water 3 times each was for 20 min. Membranes were washed with 10 x SSC for 20 min. and transferred to a Hybond N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). *LOC290876* cDNA was labeled with a random primer labeling system (GE Healthcare) using [α-^{32}P]dCTP. The membrane was hybridized with the probe, and then washed with buffer containing 1 × saline sodium citrate (SSC) and 0.1% SDS at 65°C for 20 min., and radioactivity was measured using a FLA-5000 fluorescent image analyzer (GE Healthcare). It was rehybridized with rat *Gapdh* cDNA as internal control to confirm RNA integrity and quantity.

In situ hybridization

LOC290876 expression in the testis section was analyzed by *in situ* hybridization (ISH). Riboprobes were synthesized using SP6 and T7 promoters attached to a cloned cDNA fragment of the gene as a template using DIG RNA labeling kit (Roche, Basel, Switzerland). In brief, DIG-labeled sense and antisense riboprobes were hybridized with a 5-µm section of the testis of

an 8-week-old rat. The probe was designed in the area used for RT-PCR amplification. Signals were detected using a DIG Nucleic Acid Detection Kit (Roche Applied Science, Tokyo).

Western blotting and immunohistochemistry

Protein expression was measured by Western blotting. Two synthetic oligo-peptides, C+ASEKNHQPSQDGTPL and CIRKLLRKELDSEDQS, were used to immunize rabbits, and sera were collected as polyclonal antibodies by a custom antibody producing service (Operon Biotechnologies, Tokyo). Rat tissue proteins were prepared and purified using Complete Lysis-M (Roche). Proteins (30 μg of each sample) were electrophoresed on a 10% polyacrylamide gel containing sodium dodecyl sulphate, which was transferred to a Hybond C membrane (GE Healthcare). The membrane was hybridized with antisera (1:2,000) and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; Cosmo Bio, Tokyo). Bound antibody was detected using Immunoblot Western Chemiluminescent HRP Substrate (Millipore, Tokyo). Rabbit polyclonal anti-GAPDH (Gene Tex, Los Angeles, CA) was used as control primary antibody (1:2,000).

Protein expression in the testes was analyzed by immunohistochemistry. Another testis section used in ISH was hybridized with the primary antisera (1:500) used for Western blotting, and signals were visualized using the Histofine DAB Substrate kit (Nichirei, Tokyo) following the manufacturer's protocol.

2.4 Results

RT-PCR and Northern blotting

LOC290876 gene expression was analyzed by RT-PCR and Northern blotting. The mRNA size of the gene was 1,454 bp, and the Northern blotting result confirmed this gene size. Two strong bands of similar molecular size were detected in the 7-week to 15-week-old rat testes, and expression in the 9-week-old rats was testis-specific. The band sizes were slightly smaller than those of 18S rRNA (Fig. 2-1A), and suggest the presence of splicing variants. To clarify this, PCR primer combinations were designed, which covered the entire cDNA area. PCR amplification using primers in exons 6 and 9 produced two bands at 489 bp and 373 bp (Fig. 2-1B). No multiple amplifications were observed using other primer combinations. We determined the sequences of the two fragments, which we named *LOC290876-FL* (full length isomer) and *LOC290876-TL* (truncated isomer). *LOC290876-TL* contained a 116-nucleotide deletion at the 5' boundary of exon 8 (Fig. 2-2). Part of the *LOC290876-TL* isomer sequence is to appear in the DNA Data Bank of Japan (DDBJ) under accession no. AB714639. The two isomers encoded a common sequence of 216 amino acids at the amino termini, while the carboxyl termini showed differences in amino acid length (21 versus 24 amino acids). The *LOC290876-TL*-encoded carboxyl terminal 24 amino acid sequence, deduced from the DNA sequence by BLAST survey, had no homology with the other proteins deposited in database.

Protein localization

The Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences (PSORT) program (http://psort.hgc.jp/) is commonly used to as to the presence of the signal peptide and subcellular localization of novel proteins by applying the predicted amino acid

sequences [97]. By this analysis, we found no signal peptide in the protein, suggesting that the encoded protein is not secreted. Instead, it is possible that it is a soluble protein localized in the nucleus.

Western blotting

The deduced coded protein of LOC290876-FL was determined to be 237 amino acids long. It was an acidic protein (pI 4.8) with three sphingosine 1-phosphate receptor type 5 (EDG-8) motifs according to the Ensembl survey (www.ensembl.org). Protein expression at various developmental stages of the testes and in various organs of the 9-week-old rats was determined by Western blotting. Two bands of almost the same size (26.6 kDa and 26.2 kDa) and intensity were increased at the 7-week-old rat testes. Expression was testis-specific and remained at almost same level until 15 weeks (Figs. 2-1C).

In situ hybridization and immunohistochemistry

LOC290876 mRNA expression was analyzed by *in situ* hybridization in seminiferous tubule sections according to the seminiferous epithelial cycles (Fig. 2-3). *LOC290876* mRNA expression was widespread in the cytoplasm of the spermatocytes in the individual tubules. Protein expression of the gene product was determined by immunohistochemistry and was observed mostly in the nuclei of spermatocytes (Fig. 2-4). Both *LOC290876* mRNA and protein expression were observed at specific stages of spermatocyte development, as indicated by arrows in the figures.

2.5 Discussion

To elucidate individual biological processes and the physiological functions of organisms, it is necessary to collect information about cell-specific genes and to analyze their expression. In the case of spermatogenesis, many genes have been analyzed by RT-PCR and DNA microarray, but not all of their functions have been determined. Nevertheless, gene collection represents a tool for the diagnosis of azoospermia and of specific cancers, several cancer-testis (CT) antigens which are expressed in specific cancer cells, and expression of them is restricted to developing testes in normal organs [58].

Interestingly, it is important to elucidate the biological functions of these so-called orphan genes, including that of *LOC290876*, which was found in the present study to contain three EDG-8 motifs according to the ENSEMBL survey. Ten specific elements have been identified in the EDG-8 (sphingosine 1-phosphate receptor type 5) sequence, and three of them are located in *LOC290876* product sequences. These three domains have related sequences in EDG-8: e.g., 1 lies in the N-terminus; 2 spans the second cytoplasmic loop and leads into transmembrane domain; and 3 lies in the third cytoplasmic loop. The mRNA size of *LOC290876* has been reported to be 1,454 bp, in agreement with the results of Northern blotting in this study. Rat *EDG-8* cDNA size was reported to be 2,171 bp (accession no. NM_021775). Sphingosine 1-phosphate is known to be involved in several biological processes, including mitosis and differentiation, and may be involved in cancer and angiogenesis [98]. Sphingosine 1-phosphate receptors are G-protein coupled membrane proteins and these proteins are divided into subtypes that show organ-specific expression [99]. For example, higher expression of EDG-8 occurs in the adult rat brain and spleen as compared with low expression in the testes [100]. Since no expression of *LOC290876* mRNA was detected in the adult rat brain and expression was

localized to the nucleus, according to the results of immunohistochemistry, I concluded that this gene product has a function different from EDG-8.

To determine protein structure and subcellular localization, PSORT analysis was carried out. The protein was predicted to be soluble and localized in the nucleus, in good agreement with our immunohistochemical observations. Recently, Schultz-Thater *et al.* described a novel CT antigen gene, *MAGE-A10*, which encodes a protein expressed in the nuclei of spermatogonia and spermatocytes [101]. Such expression and localization characteristics are similar to the product encoded by *LOC290876*, suggesting that these proteins have similar functions, for example, as nuclear gene expression regulators.

Finally, two splicing variants of *LOC290876* have been identified. In the truncated isoform, exon 7 was joined to the AG splicing acceptor in exon 8 resulting in truncated mRNA [102]. The proteins encoded by the two isomers differed only in their amino acid sequences at the carboxyl-terminus, and the *LOC290876-TL*-encoded carboxyl terminal showed no homology with the other proteins. There are many reports of splicing variants in spermatogenesis, which appear to vary in biological function and regulation. For example, two variants have been reported in the testicular zinc finger protein (TZF) [103] and in LIM domain-containing protein genes [104], which show increased expression in maturing testes. Since the two *LOC290876* isomers share long common amino acid sequences in their amino termini and similar expression profiles, it is possible that they have both common and distinct functions in spermatogenesis, especially during the process of meiosis, since expression of them was so widespread in spermatocytes. This result is in agreement with the result of DD, in which mRNA expression was up-regulated after 7 weeks in the rat testes when pachytene spermatocytes were observed [35].

These findings suggest that the *LOC290876*-encoded gene products are not involved in sphingosine signaling, but rather have distinct roles in the nucleus during the process of spermatocyte maturation and meiosis producing spermatids. Individual gene knockdown experiment may be a useful procedure to understand the physiological function of a novel gene product, and the Cre-LoxP system is the most useful tool to knock down the expected gene in a cell-specific manner. Germ cell-specific nuclear factor kappaB knockdown mice have been constructed [105]. *LOC290876*-encoded gene isoforms are expected to be nuclear localized. This knockdown procedure is a promising tool to understand the importance of the isoforms, which may have biological functions in spermatogenic cell development.

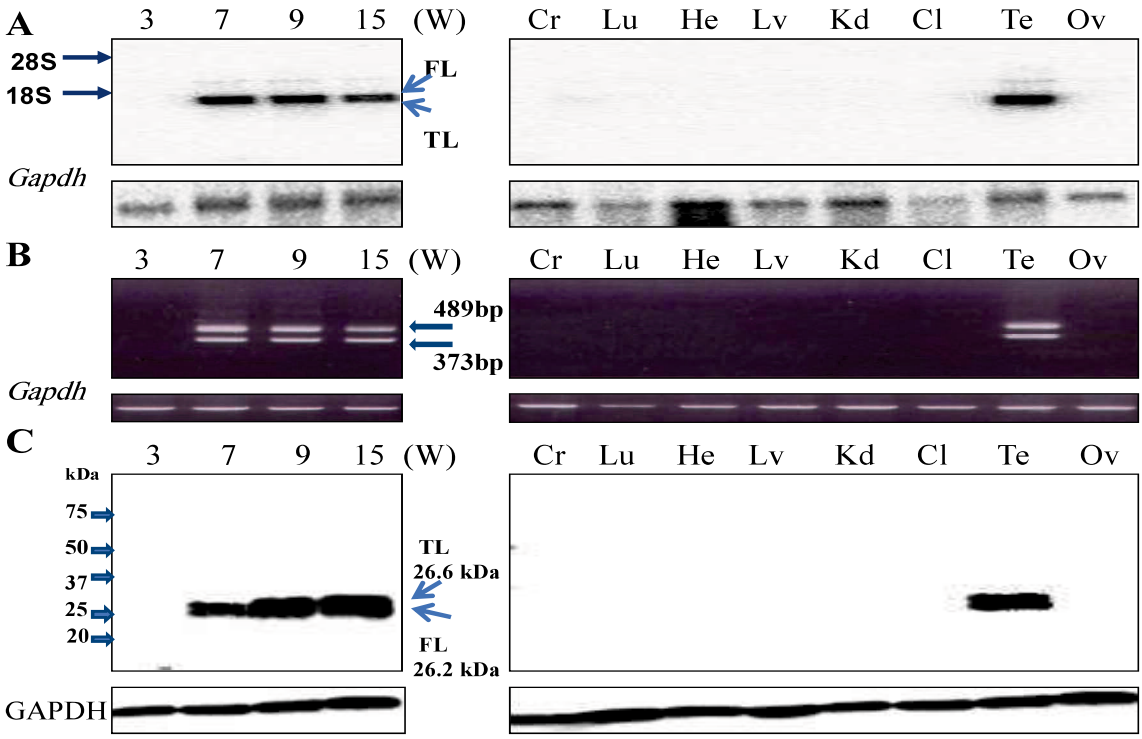

Fig. 2-1. *LOC290876* Transcript and protein analysis

A, Northern blot of *LOC290876*. Stage specific expression in 3-, 7-, 9-, and 15-week (W)-old rat testes, and organ-specific expression in 9-week-old rats (Cr, cerebrum; Lu, lung; He, heart; Lv, liver; Kd, kidney; Cl, colon; Te, testis; Ov, ovary). Five μg of RNA was loaded per lane. The positions of the rRNA are indicated by arrows on the left, as 28S (28S rRNA) and 18S (18S rRNA). The *LOC290876* mRNA signal position is indicated by arrows as FL (full length) and TL (truncated). *Gapdh* expression is indicated at the bottom. **B**, Semi-quantitative RT-PCR analysis of the exon 8 region and expression of two *LOC290876* isoforms. The sample order of testis stages and individual organs is the same as that of Northern blotting. *Gapdh* expression is indicated at the bottom. The longer FL amplification fragment is 489 bp, and the shorter TL amplification fragment is 373 bp (arrowed). **C**, LOC290876 protein analysis by Western blotting. Thirty μg of protein was loaded per lane. The positions of the 26.6 kDa and 26.2 kDa protein signals (encoded by TL and FL respectively) are arrowed. Anti-GAPDH antibody was used as internal control, as shown at the bottom.

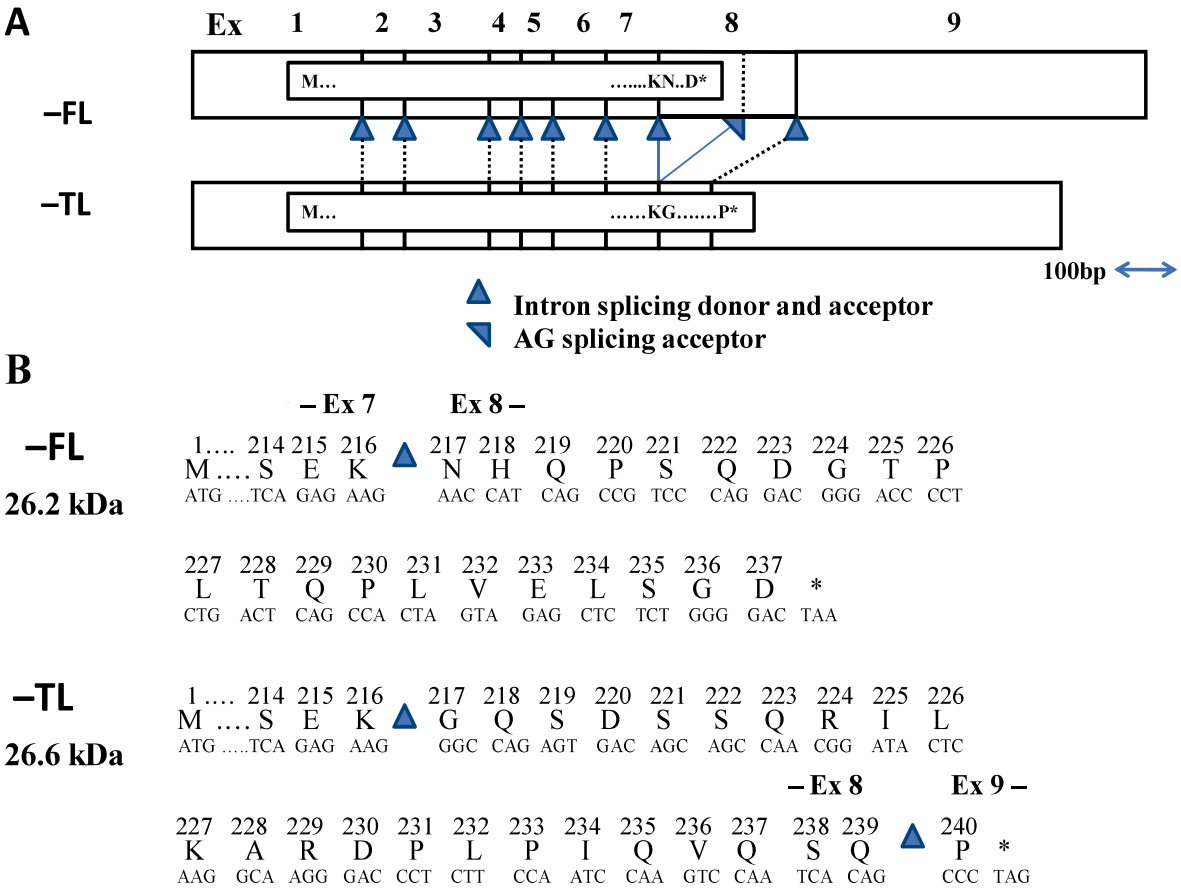

Fig. 2-2. *LOC290876* Transcript analysis

A, Expected mRNA isoforms *LOC290876*-FL (accession no. NM_001037182) and *LOC290876*-TL (accession no. AB714639) are illustrated. The *LOC290876* gene had nine exons (Ex), and *LOC290876*-TL mRNA had a deletion of 116 nucleotides in the 5' boundary of exon 8. The presence of splicing consensus signals in introns (GT or GC-donor and AG-acceptor) and the AG-acceptor in exon 8 are shown by triangles. **B**, Nucleotide and deduced amino acid sequences of *LOC290876*-FL and *LOC290876*-TL encoded regions. The amino acid sequences of the carboxyl termini differed after the lysine (K) 216 codon (FL: 21 amino acids, TL: 24 amino acids). The boundaries of exons 7, 8, and 9 are indicated by triangles. Stop codons are indicated by asterisks.

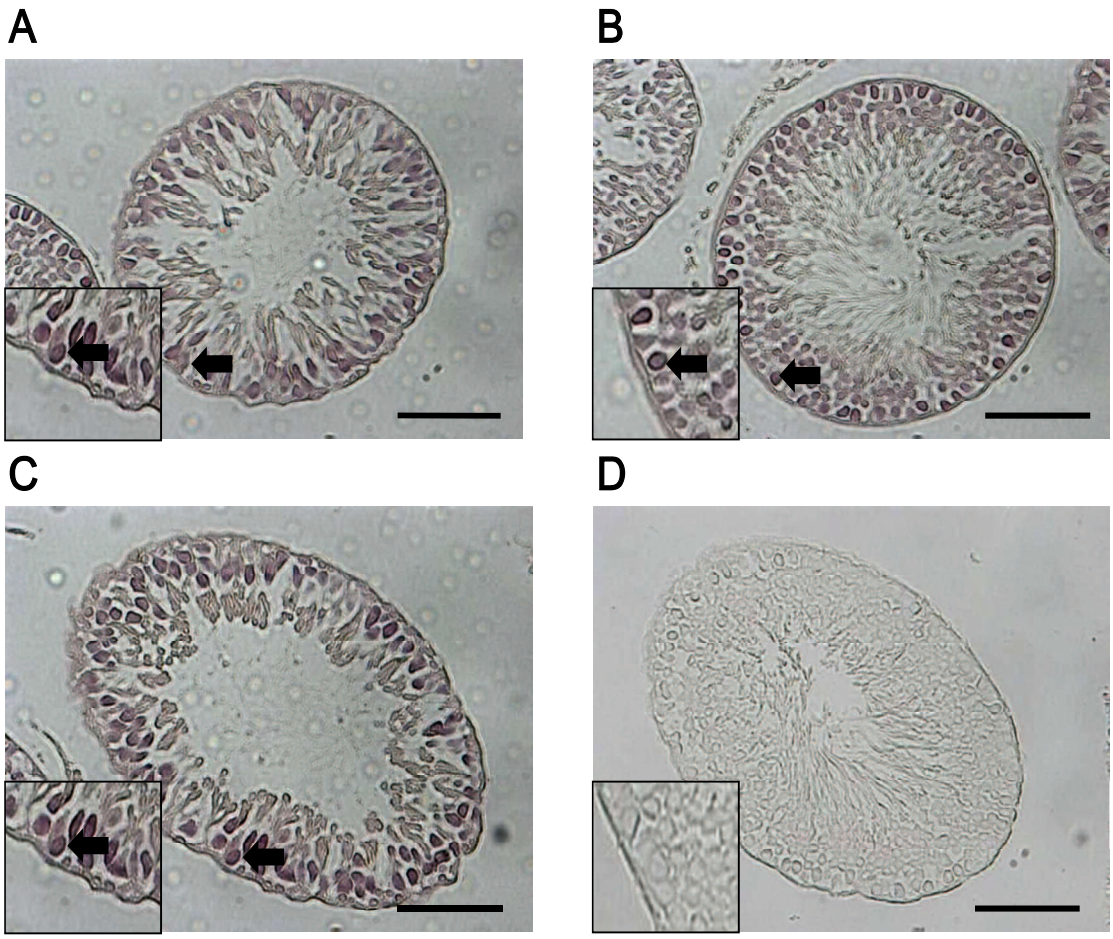

Fig. 2-3. Cellular localization of the rat *LOC290876* transcript in 8-week-old rat testes

In situ hybridization of the *LOC290876* gene product at various stages in 5-μm-thick sections of seminiferous tubules of 8-week-old rat testes (A) to (C), and using a sense riboprobe as negative control (D). Representative tubules were selected and were expected to correspond to stages I to IV (A), stages VII to VIII (B), and stages IX to XI (C). Signals (purple) in the cytoplasm of the spermatocytes (arrows) can be observed. High magnification pictures from individual tubules are given in boxes. Scale bar, 100 μm.

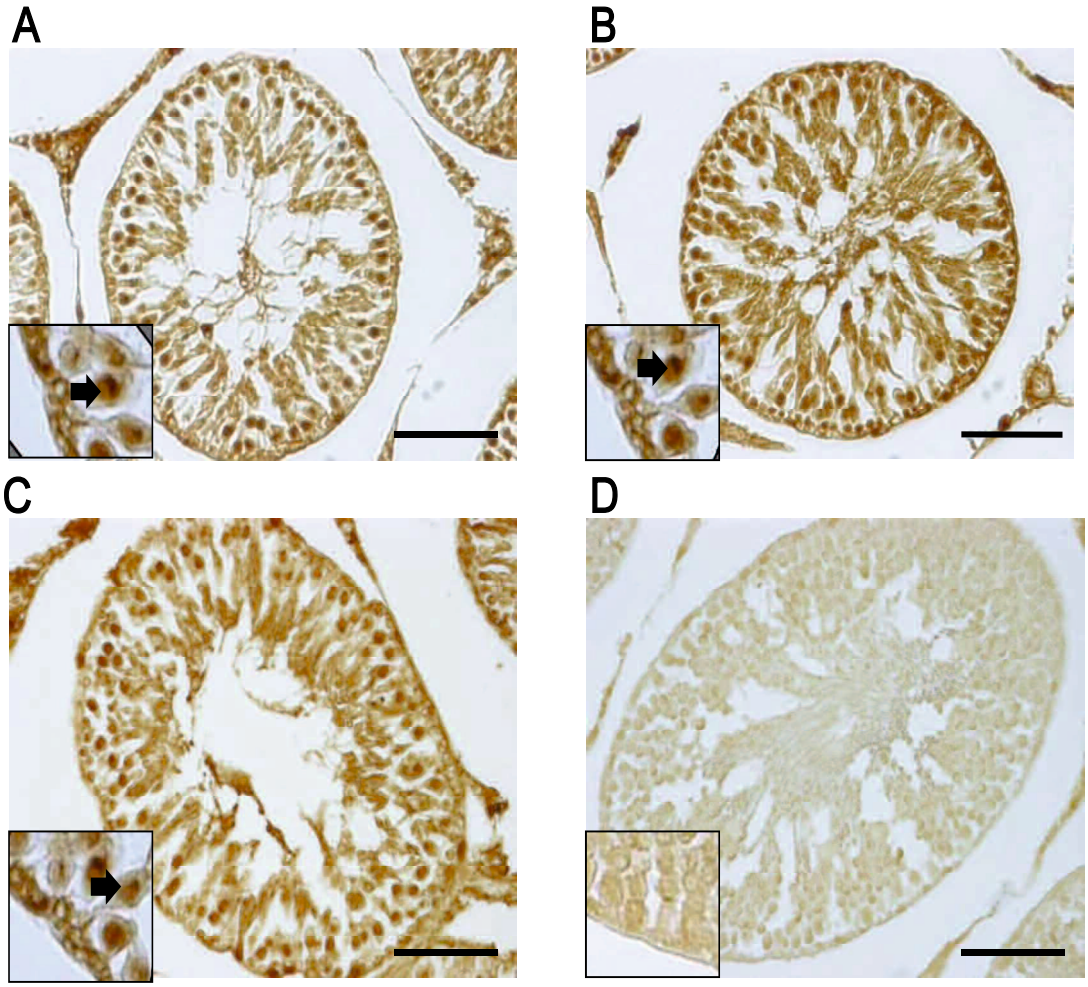

Fig. 2-4. Cellular localization of rat LOC290876 gene product in 8-week-old rat testes

Immunohistochemistry using rat LOC290876 gene product-derived oligopeptide-raised antisera at various stages on 5-μm-thick sections of seminiferous tubules from 8-week-old rat testes (A) to (C), with pre-immune serum as negative control (D). Tubules were expected to correspond to stages I to IV (A), stages VII to VIII (B), and stages IX to XI (C). Signals (brown) were observed in the spermatocytes. High magnification pictures from individual tubules are given in boxes. Signals in nucleus are arrowed. Scale bar, 100 μm.

Chapter III

Rat Stem-Cell Leukemia Gene Expression Increased during Testis Maturation

3.1 Abstract

Stem-cell leukemia (SCL), also known as T cell acute-lymphocytic leukemia (*Tal-1*) gene expression, was up-regulated in the maturing rat testis in this study. Strong expression of *Tal-1* was detected in the normal maturing rat testis by Northern blotting. Western blotting revealed the protein size to be about 34 kDa. Protein expression was wide-spread in spermatocytes, spermtids and spermatogonia in accordance with the seminiferous epithelium cycle, as determined by an analysis of immunohistochemistry. Gene expression of *Tal-1* regulatory gene, *NKX3.1*, was negatively correlated with *Tal-1* expression. Human *Tal-1* expression in the maturing testis as well as in bone marrow was observed, which suggests that the gene product is a novel cancer-testis antigen candidate. Taken together, TAL-1 may be involved in cell division, morphological changes, and the development of spermatogenic cells in the normal rat testis.

3.2 Introduction

Spermatogenesis is regulated by the expression of many genes, and morphological changes in spermatogenic cells and their maturation proceed systematically during this process. In this study I have cloned and characterized some of these genes by differential display (DD), and categorized them according to expected functions [53-58]. Of these, tumor antigens commonly expressed in normal testis are interesting, because they can be used for cancer diagnosis, and are promising tools for cancer immunotherapy [85-87] and can be used to understand the process of spermatogenesis. Colon cancer specific antigens have been screened by a recombinant cDNA expression library (SEREX) from an individual cancer patient, and they are promising candidates as cancer-testis (CT) antigen genes [88]. The rat homolog of the serologically defined colon cancer antigen 8 gene (*Sdccag8*), have been identified, which was screened by SEREX, and expression was rat testis specific [58]. The gene product was identified as serologically defined colon cancer antigen 8 (SDCCAG8) and centrosomal colon cancer autoantigen protein (CCCAP), and its C-terminal portion was identical to the serologically defined human colon cancer autoantigen (NY-CO-8) [88, 89]. According to this result, we consider it a rat CT antigen candidate.

T-cell acute-lymphocytic leukemia (T-ALL) is a malignant blood stem-cell cancer commonly observed in children. In T-ALL, blood stem cells do not differentiate into mature blood cells, such as erythrocytes or white blood cells, and patients have severe anemia, are vulnerable to infectious diseases, and usually have a poor prognosis. Novel CT antigen candidates are therefore promising tools for diagnosing T-ALL. One such candidate, TSGA10, has been reported. Its expression was observed in several cases of cancers especially acute lymphoblastic

leukemia, using quantitative RT-PCR for bone morrow samples. It might be involved in the mitotic checkpoint during T-ALL development [106]. Interestingly, searching for other leukemia CT antigens, and recently I studied the expression of the T-cell acute-lymphocytic leukemia (*Tal-1*) and the stem-cell leukemia (*SCL*) gene in maturing rat testes. Baer identified a *Tal-1* gene rearrangement in T-ALL patients due to chromosomal translocation and DNA recombination. [107].

Kallinapur *et al.* determined gene and protein expression in neonatal and adult mice. Normal *Tal-1* expression in mouse embryogenesis has been observed in the yolk sac blood island, and in the fetal liver, spleen, and thymus, and in developing vascular system, brain, and cartilage [108]. *Tal-1* deletion mutants suggest that the gene functions in angiogenesis and hematopoietic differentiation, such as bone marrow erythropoiesis during embryogenesis, as well as in T-ALL development [109-111]. Murell *et al.* observed increased expression of SCL in the mRNA and protein levels during erythroid differentiation [109]. Porcher *et al.* suggested the essential role of *Tal-1* for development of all hematopoietic lineages; furthermore, they reported the early requirement of *Tal-1* in hematopoietic development [110]. To confirm the important role of *Tal-1*, *Robb et al.* generated embryonic stem lines with homozygous null mutation of *Tal-1* gene. Their observations revealed the importance of *Tal-1* in definitive hematopoiesis and formation of hematopoietic cell [111].

TAL-1 also functions with GATA1 and LIM transcription factors during erythropoiesis [112, 113], and is an important basic helix-loop-helix (bHLH) transcription factor expressed in developing erythroid cells [114]. Shivdasani *et al.* showed the important role of *Tal-1* for embryonic blood formation *in vivo*. *Tal-1* deficiency resulted in loss of the erythroid

transcription factor GATA-1 or the LIM protein during embryonic erythropoiesis [112]. Further report mentioned about the essential role of the bHLH family including TAL-1 which function sequentially, cooperatively, or antagonistically during differentiation of erythroid cells [114].

Chromatin immunoprecipitation (ChiP) sequencing analysis has been used to identify TAL-1 target genes [115-119], which include tumor suppressor genes such as *NKX3.1* [120], stem cell regulators such as *Gfi1* [121], and specific adhesion molecules such as *VE-cadherin* [122]. NKX3.1 is necessary for T-ALL proliferation which partially restored the effect of TAL-1 knockdown cells [120]. Wilson *et al.* studied the regulation of *Gfi1* expression, which functions as key regulator of stem cell hematopoiesis, they reported five upstream regulatory regions including *Tal-1* [121]. Moreover, Deleuze *et al.* identified *VE-cadherin* as a target gene for TAL-1 complex supporting the involvement of TAL-1 in angiogenesis [122]. The TAL-1 complex binds the E-box-associated GATA motif, and this interaction can modify gene expression during angiogenesis and blood cell development. A combination of TAL-1 and LIM domain protein LMO2 has been found to lead to the development of T-ALL [123].

Human *Tal-1* expression is largely limited to the fetal liver and to adult bone marrow, brain, and cartilage. TAL-1 protein expression in tissues and cell lines has been analyzed, but expression in the developing testis is not determined [124, 125]. To evaluate the importance of TAL-1 expression during testis development, I examined it in the maturing rat testis and determined its function in relation to spermatogenesis.

3.3 Materials and Methods:

Animals and samples preparation

Sprague-Dawley (SD) rats were purchased from CLEA Japan (Tokyo). All rats were sacrificed by ether inhalation; organs were quickly dissected, removed and kept in -80 °C for further investigation. Total RNA from testes at various developmental stages and from individual organs of 9-week-old male rats and a female rat was prepared using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. A bone marrow cell suspension was harvested by flushing the cavity of the femurs and tibiae with phosphate-buffered saline, the cells were collected by centrifugation, and total RNA was prepared. Human total RNA was purchased (Takara Bio, Shiga, Japan). Fetal liver RNA was prepared from 63 spontaneously aborted male and female Caucasian fetuses (ages 22-40 weeks), liver RNA was from a 51-year-old male Caucasian, bone marrow RNA was from 56 Asian males and females (ages 22-95), and testis RNA was from five Asians (ages 21-29). cDNA was synthesized from 1 μg of RNA using a first-strand cDNA synthesis kit including Super Script III Reverse Transcriptase (Life Technologies). All animal experiments were approved by the Institutional Animal Care and Use Committee of Tottori University.

Semi-quantitative RT-PCR

PCR was performed using *TaKaRa Ex Taq* DNA polymerase (Takara Bio) under the following conditions: 94°C for 5 min., then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min., followed by extension at 72°C for 10 min. The PCR amplification primers were as follows: rat *Tal-1* sense primer 5'- GGTTGGAAATGATGGCCAGTAACC-3' and antisense primer 5'- AACAACTGGTCAGGCAGAATCCC-3'(accession no. NM_001107958), rat

NKX3.1 sense primer 5'- TCTGCTTCAGAGCTCGACGAA-3', and antisense primer 5'-AGCTAGCATACACGGAGACCAA-3' (accession no. NM_001034144), rat *Gfi1* sense primer 5'- GGACAAGAGTGTGGGAGTCAAGG-3' and antisense primer 5'-GGGCTTGAAACCTGTGTGCTTTC -3' (accession no. NM_012566), rat *VE-cadhelin* sense primer 5'- CCACTATGTGGGAAAGATCAAGTCC-3' and antisense primer 5'-TCTTGTGCTTCCACCACGATCT-3' (accession no. NM_001107407), and human *Tal-1* sense primer 5'- CAAGCCTCGAGGTGCCTTCT-3' and antisense primer 5'-GGACAGGTTTTGTGCTCAATTCGG-3' (accession no. NM_003189). Mammalian *Gapdh* primers 5'- AGTCGGAGTGAACGGATTTGG -3', and antisense primer 5'-AGTTGTCATGGATGACCTTGG -3' were used for PCR amplification as internal control. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

Northern blotting

For Northern blotting, 5 μg of total SD rat RNA was electrophoresed on a formaldehyde-containing 1% agarose gel in 1X MOPS after incubation at 65 °C for 15 min. for the RNA samples. After electrophoresis, the membranes were stained using ethidium bromide for 20 min. shaking and then washed using distilled water 3 times each was for 20 min. Membranes were washed with 10 x SSC for 20 min. and blotted onto a Hybond N^+ nylon membrane (GE Healthcare, Buckinghamshire, UK). The *Tal-1* cDNA probe was labeled by a random primer labeling system (GE Healthcare) using [α-^{32}P] dCTP. The membrane was hybridized with the probe, and then washed with buffer containing 1 × saline sodium citrate (SSC) and 0.1% SDS at 65°C for 20 min., and radioactivity was measured using a FLA-5000 fluorescent image analyzer (GE Healthcare). It was rehybridized with rat *Gapdh* cDNA as internal control to confirm RNA integrity and quantity.

Cloning and sequencing of the Tal-1 upstream region

The rat *Tal-1* cDNA sequence was deposited in the DNA database under accession no. NM_001107958. It encodes a predicted 212-amino-acid protein, but the upstream sequence of the proposed start methionine codon was different from that of mouse and human. To clarify the upstream sequence, we designed primers for PCR amplification in a 5′direction from the proposed start codon based on conserved sequences of mouse and human. The designed upstream sequence primer was 5'- GGACCTCACGGCAAGCTAAG-3', and the downstream sequence primer was 5'- GCTAAGGCTATAGAGCAGCGC-3'. PCR was performed using *Tks Gflex* DNA polymerase (Takara Bio) according to the following protocol: 94°C for 1 min, then 34 cycles of 98°C for 10 s, 60°C for 15 s, and 68°C for 1 min, followed by extension at 68°C for 10 min. Amplified cDNA, in which A-overhangs were introduced, was cloned into pGEM-T Easy vector and sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Tokyo). The DNA sequence was compared to that of *Tal-1* cDNA sequences deposited in the DNA database.

***In situ* hybridization**

A riboprobe designed using the same sequence as the rat *Tal-1* RT-PCR amplified fragment was synthesized using a DIG RNA Labeling kit (Roche, Basel, Switzerland). A 5-μm section of 8-week-old rat testis was hybridized with the probe, and the signal was developed using a DIG Nucleic Acid Detection Kit (Roche) following the manufacturer's instructions. The sections were counterstained with methyl green.

Western blotting and immunohistochemistry

Rat tissue proteins were prepared and purified using Complete Lysis-M (Roche Applied Science, Tokyo), and 10 μg of each protein sample was electrophoresed on a sodium dodecyl sulphate 10% polyacrylamide gel (SDS-PAGE), and transferred to a Hybond C membrane (GE Healthcare). The membrane was hybridized with TAL-1 antisera (QC1680, Aviva Systems Biology, San Diego, CA) (1:2,000) and incubated with HRP-conjugated goat anti-rabbit IgG (Cosmo Bio, Tokyo) (1:10,000) as secondary antibody. Bound antibody was detected using Immunoblot Western Chemiluminescent HRP Substrate (Millipore, Tokyo). Rabbit polyclonal anti-GAPDH (Cosmo Bio) was used as control primary antibody (1:2,000).

The sections used in ISH were hybridized with the same primary antisera (1:100) as used in Western blotting, and the signals were detected using a Histofine DAB substrate kit (Nichirei, Tokyo) following the manufacturer's instructions.

3.4 Results

RT-PCR, Northern blotting, and Western blotting

DD have been used to screen for genes expressed specifically during the sexual maturation of the rat testis, and focused on one candidate, *Tal-1* (*SCL*). Gene expression was determined by semi-quantitative RT-PCR and Northern blotting. *Tal-1* gene expression was observed as a 458 bp specific band in RT-PCR and a 28S rRNA-size band in Northern blotting. Expression was up-regulated during testicular development, reaching maximum levels in 7-week-old and older rats (approximately a 6-fold increase from 3 weeks to 7 weeks of age). Strong expression of *Tal-1* was detected in the maturing rat testis, and expression in the normal bone marrow was about one quarter the intensity of the testis according to the Northern blotting results, although faint expression was observed in the lung, heart, and cerebrum of the 9-week-old rats by RT-PCR (Fig. 3-1A, B). TAL-1 protein expression was determined by Western blotting, and was found to be weak in the testes of the 3-week-old rats, increasing after 7 weeks of age and remaining stable thereafter. The expression level in the testis was almost same as that of the bone marrow. The protein size was approximately 34 kDa (Fig. 3-1C).

TAL-1 regulatory gene expression was measured by semi-quantitative RT-PCR. Although *Gfi1* expression was thymus-specific and *VE-cadherin* expression was wide spread among the mature rat organs, *NKX3.1* expression was testis-specific, and the level was highest in 3-week-old and decreased thereafter (Fig. 3-2A). The expression of human *Tal-1* was high in the fetal liver, adult bone marrow, and testis, but expression in adult liver was weak (Fig. 3-2B).

Cloning and sequencing of the Tal-1 upstream region

The 369-bp cDNA sequence 5' upstream from the *Tal-1* methionine codon was determined, and was deposited in the DNA sequence database under accession no. AB728506. A comparison of rat *Tal-1* cDNA sequence we determined (rat-TE) with that deposited in database (rat-DB) and the mouse *Tal-1* cDNA sequence database (mouse) revealed an in-frame stop codon between two methionine codons (tga) in the rat-DB that was absent from the rat-TE. In the rat-TE, an in-frame stop codon (taa) was observed upstream of the 5' methionine codon (Fig. 3-3). The deduced amino acid sequences between the two methionine codons of mouse and rat-TE were found to share high homology (93.2%).

In situ hybridization and immunohistochemistry

Gene expression in the rat testis section was determined by ISH, and was found to be high in spermatocytes (Fig. 3-4). Immunohistochemistry revealed that TAL-1 protein expression occurred in the nuclei of the spermatocytes and speremtids, including the spermatogonia, according to the seminiferous epithelial cycle. Expression was observed in the nuclei of the spermtids during stages VII and VIII (Fig. 3-5).

3.5 Discussion

CT antigens are powerful tools for cancer diagnosis and treatment. They are expressed in specific tumors, and normal organ expression is restricted to male germ cells, not adult somatic cells, even though the reason for this expression profile is not fully understood [87]. DD have been used to screen maturing rat testis-specific genes, focusing on novel CT antigen candidates related to T-ALL. Since T-ALL diagnosis and treatment of the younger generation is a high priority, T-ALL related CT antigens are urgently sought. Expression of TAL-1 was previously confirmed to be higher in the bone marrow cells of a pre-leukemia patient, [126], and *Tal-1* gene rearrangement has been observed in T-ALL patients [127]. On the basis of this knowledge, the possibility of using the *Tal-1* gene as a CT antigen candidate has been evaluated.

TAL-1 has a bHLH domain and can be a positive regulator of several downstream genes expressed in the bone marrow in relation to the development of T-ALL. *Tal-1* gene expression was strong in the rat testes after 7 weeks of sexual maturation. Stronger expression of *Tal-1* in the bone marrow and in the testis, and faint expression in the lung, heart, and cerebrum in mature rats was detected, and it is known that faint expression of CT antigens occurs in many normal organs by sensitive RT-PCR analysis [87], even though the reason for the expression has not been documented. *Tal-1* expression in the developing testis has not been reported to date [124, 125] and strong expression in human as well as in rat was observed in this study. According to these results, we consider that this gene product can be classified as a novel CT antigen candidate, functionally related to stem-cell development. Rat *Tal-1* mRNA approximated 28S rRNA size, in agreement with the size in the DNA database (4,297 bp).

Western blotting detected a 34 kDa protein in the developing testis, bone marrow, and lung. Even the meaning of weak expression in lung is not understood. Protein signals in the kidney, colon, spleen, and thymus were larger than 34 kDa and can be considered non-specific. The rat *Tal-1* mRNA sequence was deposited in the DNA sequence database under accession no. NM_001107958, in which the predicted 212 amino acid long protein is reported. Since the expected protein molecular weight was less than 34 kDa, we analyzed the upstream sequence of the proposed start methionine codon, considering the existence of the longer open reading frame (ORF). Rat upstream cDNA sequencing revealed no in-frame stop codons between the two methionine codons and the presence of an in-frame stop codon 5′ upstream of the methionine codon in Rat-TE. In this upstream region, the rat-TE DNA sequence was different from the Rat-DB in our experiment. As the sequenced region was present in one exon of the genome information and we amplified this area as a single band by PCR, we concluded that rat-TE is not a splicing variant of rat-DB DNA. Accordingly, the rat *Tal-1* ORF was determined to be 329 amino acids long and the TAL-1 molecular weight was predicted to be 34 kDa, in good agreement with our Western blotting results.

A novel finding of this study is the analysis of TAL-1 expression in the developing testis, which was widespread depending on the seminiferous epithelial cycle in the nucleus of the spermatogonia as well as the spermatocytes and spermatids. It is plausible that weak expression of *Tal-1* was observed in the 3-week-old rat testis according to our results of Northern blotting, because at this stage pachytene spermatocytes appear [35]. The results of *in situ* hybridization indicate that the major *Tal-1* expression was in the spermatocytes. It is remarkable that the translated TAL-1 protein in the spermatocytes was stably maintained in the spermatids. Since TAL-1 is expected to function as a transcription factor, the observed nuclear localization of the

protein was plausible. Spz1 is another bHLH transcription factor, known to be specifically expressed in the testis that plays a role in the MAPK signaling pathway [128, 129]. *Tal-1* was expressed in the bone marrow as well as in the developing testis, and expression of it has been reported to be up-regulated in pre-leukemic disorder, [126] suggesting that it might be a transcription regulator of commonly activated genes among spermatogenic, bone marrow, and T-ALL cancer stem cells. Several TAL-1 target genes, including tumor suppressor *NKX3.1*, might participate in T-ALL development [120]. *NKX3.1* is a homeobox gene, that might be deeply involved in prostate development, and loss of its function is related to prostate carcinogenesis [130]. The *Tal-1* and *NKX3.*1 expression profiles in the testis were negatively correlated in our experiment. It may be hypothesized that *Tal-1* down-regulates gene expression of *NKX3.1* in the maturing testis, leading to spermatogenic cell division and differentiation.

Future work ought to clarify gene expression regulation taking into account common mechanisms in stem cell development and differentiation, leading to erythropoiesis, carcinogenesis, and spermatogenesis. These results should provide an understanding of the principal mechanism of TAL-1 in spermatogenesis, as well as identifying causes and promoting the treatment of T-ALL.

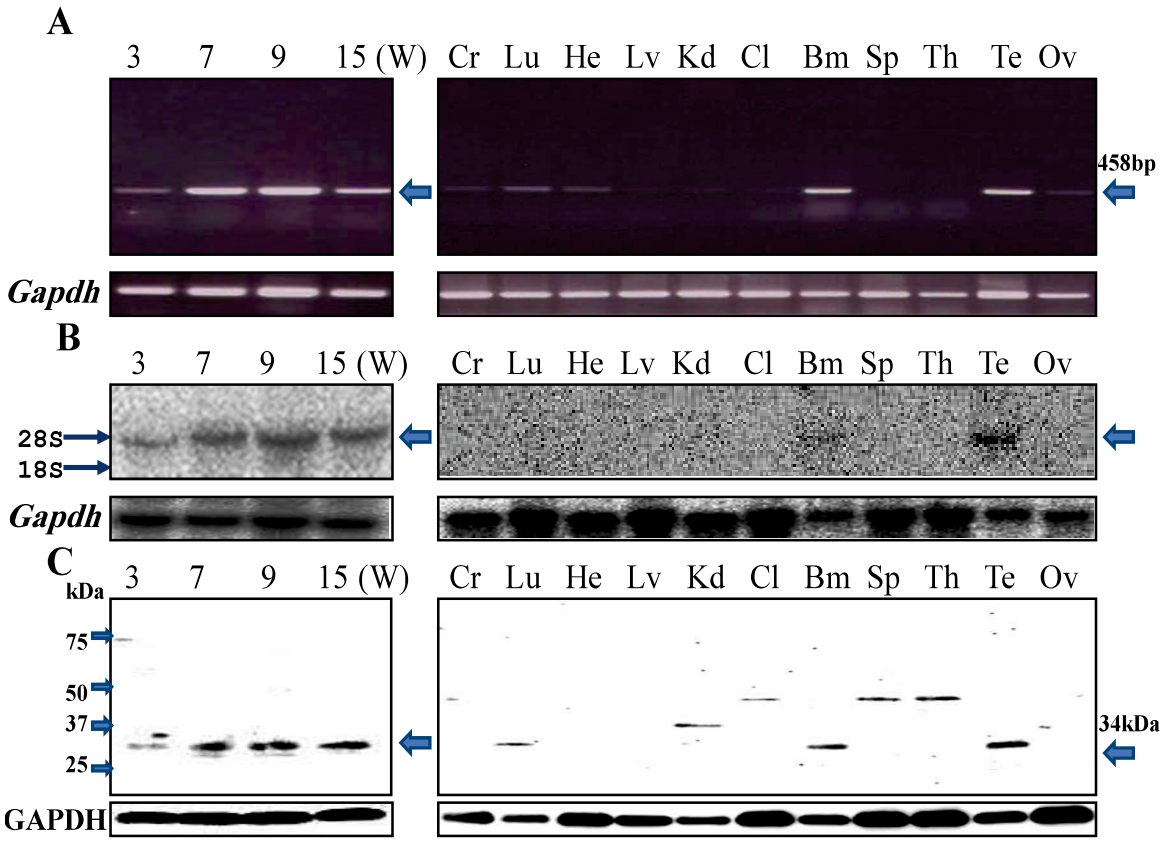

Fig. 3-1. RT-PCR, Northern blot, and Western blot analysis of rat *Tal-1* transcript and TAL-1 protein

A, *Tal-1* RT-PCR transcript analysis of 3-, 7-, 9-, and 15-week-old rat testis, and organs of 9-week-old rats (Cr, cerebrum; Lu, lung; He, heart; Lv, liver; Kd, kidney; Cl, colon; Bm, bone marrow; Sp, spleen; Th, thymus; Te, testis; Ov, ovary). Arrows indicate amplified 458-bp fragments. Rat *Gapdh* amplifications are indicated at the bottom. **B**, *Tal-1* transcript analyzed by Northern blotting. Arrows on the left indicate positions of ribosomal RNA (28S rRNA and 18S rRNA). Arrows on the right indicate *Tal-1* mRNA. Rat *Gapdh* expression is indicated at the bottom. **C**, TAL-1 protein analysis by Western blotting. Arrows on the left indicate positions of protein standards. Arrows on the right indicate TAL-1 signal position (34 kDa). The anti-GAPDH antibody signal is indicated at the bottom.

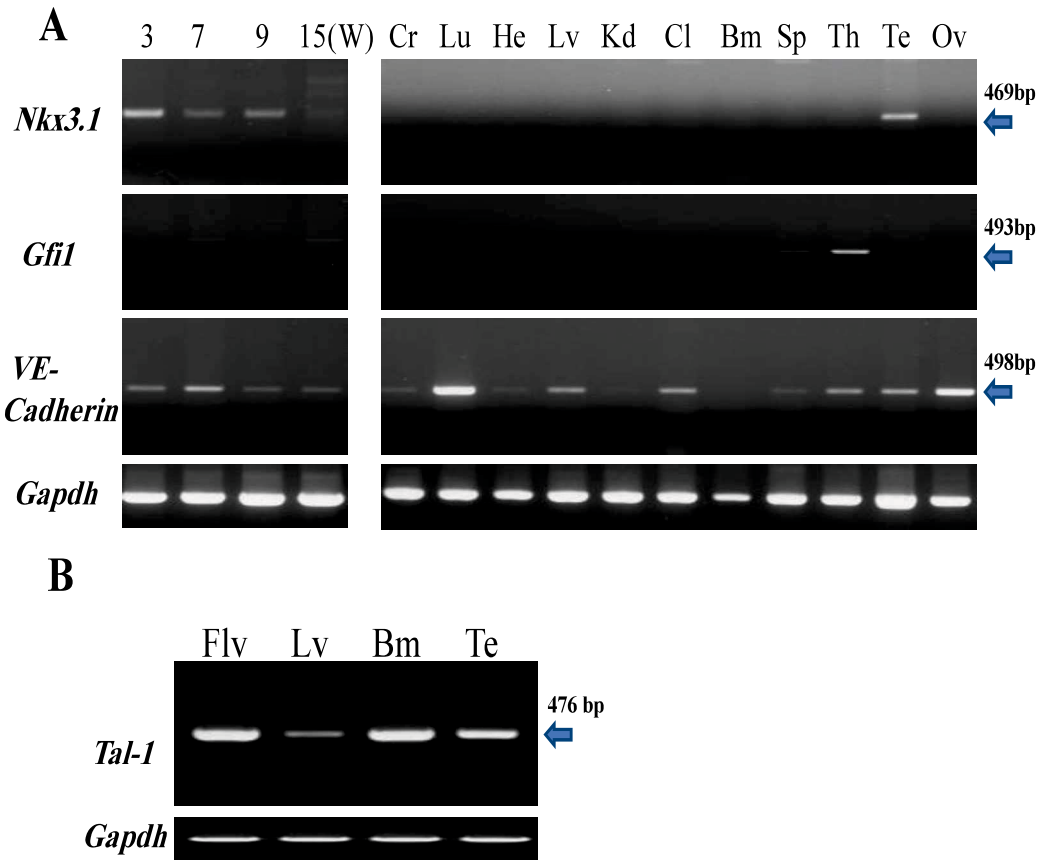

Fig. 3-2. *NKX3.1*, *Gfi1*, and *VE-Cadherin* gene expression in maturing rat testis, and *Tal-1* expression in human organs

A, *NKX3.1*, *Gfi1*, and *VE-Cadherin* RT-PCR transcript analysis of 3-, 7-, 9-, and 15-week-old rat testis, and organs of 9-week-old rats (Cr, cerebrum; Lu, Lung; He, heart; Lv, liver; Kd, kidney; Cl, colon; Bm, bone marrow; Sp, spleen; Th, thymus; Te, testis; Ov, ovary). Arrows indicate amplified fragments. Rat *Gapdh* amplifications are indicated at the bottom. **B**, *Tal-1* RT-PCR transcript analysis of human fetal liver: Flv (63 spontaneously aborted male and female Caucasian fetus, ages 22-40 weeks), adult liver: Lv (51-year-old male Caucasian), bone marrow: Bm (56 Asian males and females, ages 22-95) and testis: Te (five Asians, ages 21-29). Human *GAPDH* amplifications are indicated at the bottom.

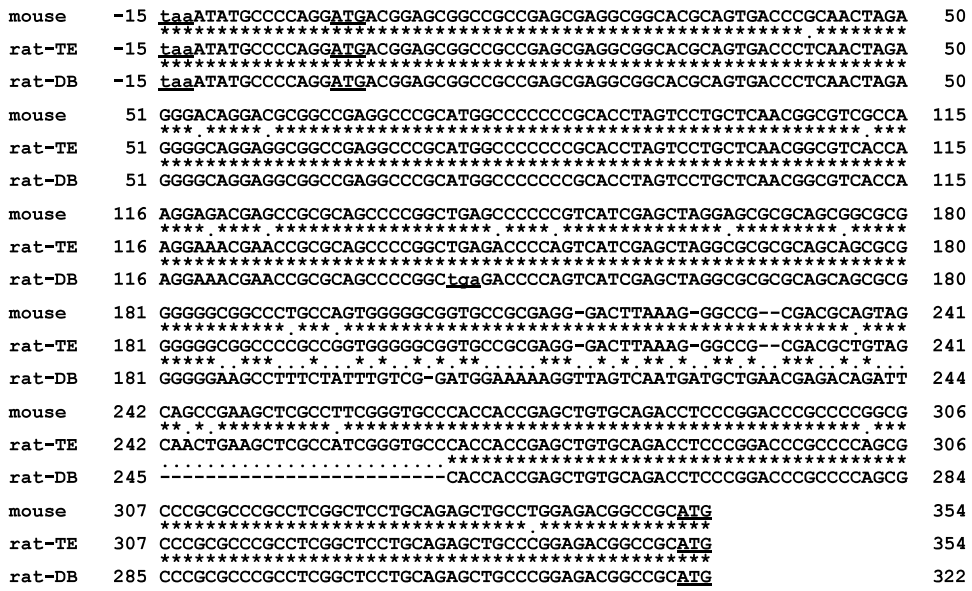

Fig. 3-3. *Tal-1 5′* Upstream cDNA sequence comparison with sequences in the DNA database

Tal-1 cDNA 5′ upstream sequence (rat-TE) was deposited in DNA database Japan (DDBJ) under accession no. AB728506. This sequence was compared with rat *Tal-1* (rat-DB: accession no. NM_001107958), and mouse *Tal-1* (mouse: accession no. NM_011527.2), and two methionine codons (underlined) in addition to the in-frame stop codons (lowercase and underlined) are indicated. Dashes (-) are introduced in the sequence to produce maximum homology. Asterisks (*) represent matching nucleotides of the rat-DB and mouse with the rat-TE cDNA sequence. Dots (.) represent non-matching bases.

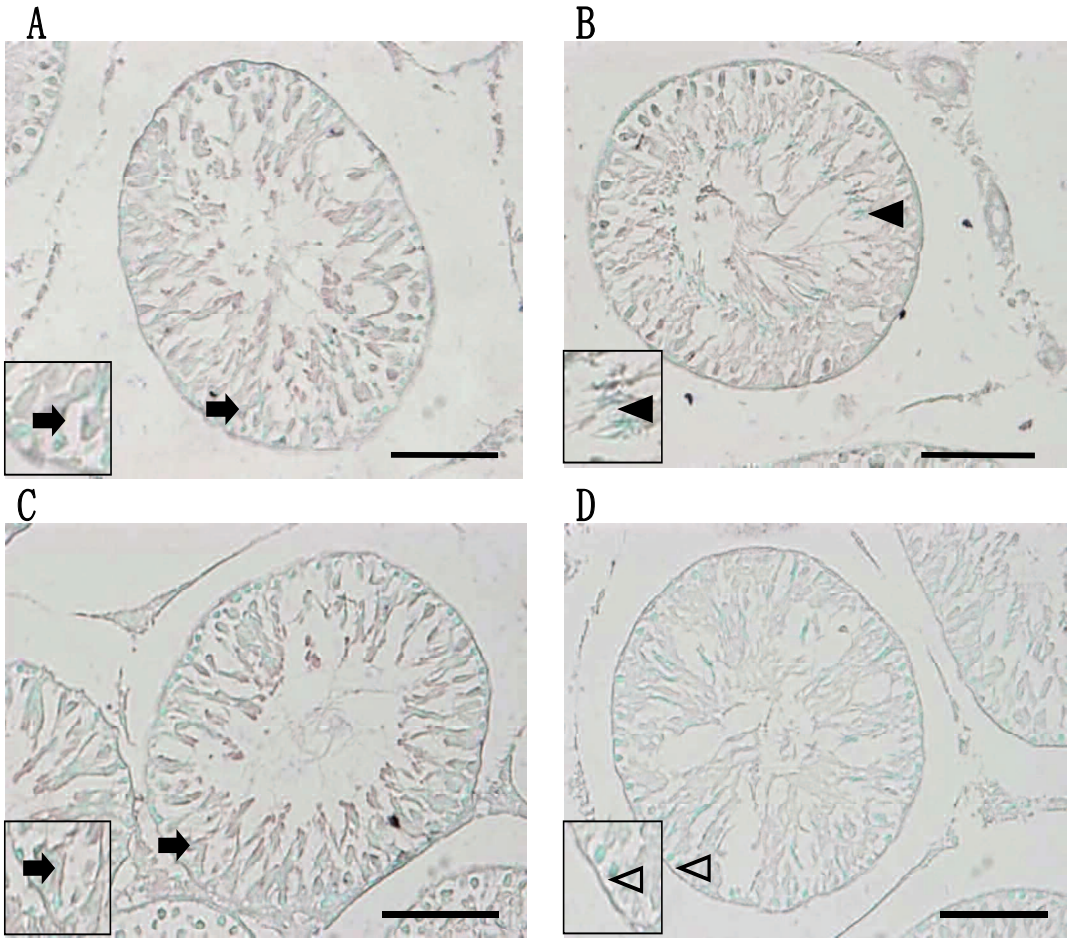

Fig. 3-4. Cellular localization of rat *Tal-1* transcript in 8-week-old rat testis

In situ hybridization using the *Tal-1* probe at various stages in 5-μm-thick sections of 8-week-old rat testis seminiferous tubules (A–C), and a sense riboprobe negative control (D). The tubules were expected to be stages I to IV (A), stages VII to VIII (B), and stages IX to XI (C). Signals (purple) in the spermatocytes (arrows) can be observed. Spermatid (arrowhead) was negative. High magnification photos of individual tubules are indicated in the boxes. The negative cell of the sense riboprobe experiment is indicated by hollow arrowheads. Sections were counterstained with methyl green. Scale bar, 100 μm.

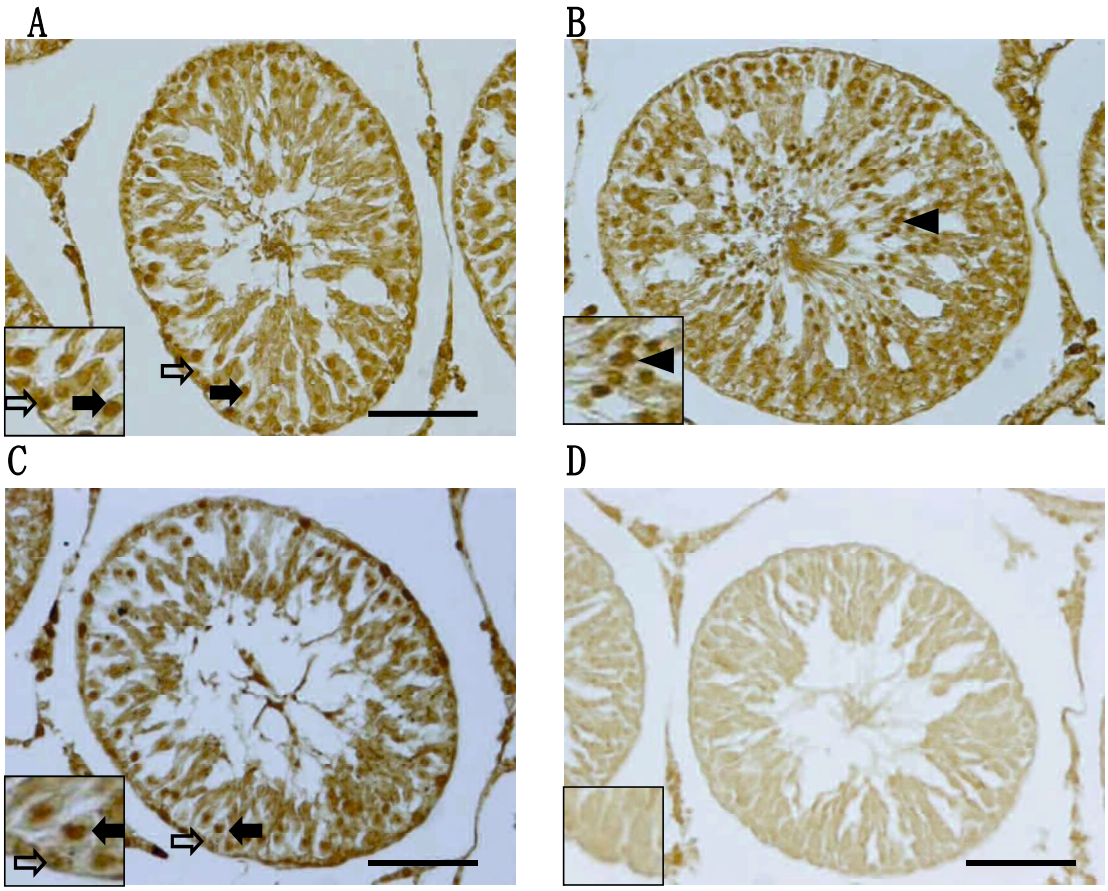

Fig. 3-5. Cellular localization of rat TAL-1 protein in 8-week-old rat testis

Immunohistochemistry using rat TAL-1 antisera at various stages in 5-μm-thick sections of 8-week-old rat testis seminiferous tubules (A–C), and pre-immune serum negative control (D). The tubules were expected to be stages I to IV (A), stages VII to VIII (B), and stages IX to XI (C). Signals (brown) in the nuclei of spermatogonia (hollow arrows), spermatocytes (arrows) and spermatids (arrowheads) can be observed. High magnification photos of individual tubules are indicated in boxes. Scale bar, 100 μm.

Chapter IV

Tumor Suppressor Candidate TUSC3 Expression during Rat Testis Maturation

4.1 Abstract

The analysis of microarray data obtained by comparing the gene expression between 2-week-old infant and 7-week-old mature SD rat testis revealed novel targets involved in tumor suppression. Reverse-transcription polymerase chain reaction and Northern blotting showed that *Tusc3* gene expression was up-regulated in the normal maturing testis and prostate and other organs such as the cerebrum and ovary. TUSC3 protein expression was detected in these same organs with a size of around 40 kDa, which agreed with the predicted molecular size. *In situ* hybridization and immunohistochemistry showed that mRNA and protein localization was prevalent in the testis spermatocytes and interstitial cells such as Leydig cells, as well as prostate epithelial cells. These data suggest that TUSC3 may be deeply involved in spermatogenesis in the testis to induce sperm differentiation and maturation and play a role in normal prostate development and tumor suppression.

4.2 Introduction

Spermatogenesis is a highly sophisticated process involved in the transmission of genetic heritage. It occurs in successive mitotic, meiotic and post-meiotic phases, and genes expressed during this process encode proteins necessary for systematic germ cell development. We previously cloned some of these genes by differential display (DD), and characterized them according to their prospective functions [53-58,131,132]. Several cancer-related gene expressions were shown to be up-regulated during sexual maturation, and are interesting targets to understand the common mechanism between carcinogenesis and spermatogenic stem cell development and differentiation. We have analyzed the expression of cancer-testis (CT) antigen genes, such as *Sdccag8*, related to colon cancer [58], and *Tal-1*, related to acute T-lymphocytic leukemia in developing rat testis [132]. They are expected to be transcription factors to regulate and stimulate downstream target gene expression, leading to carcinogenesis or proper spermatogenesis. We also found the expression of a tumor suppressor gene, such as *Hrasls5*, and the expression was up-regulated in the developing testis [57]. As the localization of the CT antigens and the tumor suppressor was observed commonly in the spermatocytes, close relation among these molecules was expected to proceed normal spermatogenesis. To clarify this point, surveying the data, considering expression profiling of other cancer suppressor candidates is urgently sought.

DNA microarray analysis has been introduced to overall screening of the genes in the developing testis, focusing on cancer suppressors, and analyzed one candidate, tumor suppressor candidate gene 3 (*Tusc3*), because the histological analysis of this gene product in the developing testis section is not clarified yet. *Tusc3* gene expression was analyzed in prostate cancer [71-74], ovarian cancer [75-77], gastric cancer [78], osteosarcoma [79], and pancreatic adenocarcinoma

[80-82] by now. High-density screening of human prostate, lung, liver, and colon tumor cell lines revealed homozygous deletions spanning the entire short arm of chromosome 8, which included the *Tusc3* gene [133]. In addition, *Tusc3* deletion was reported in lymph node tumors [83], breast and pancreatic cancer [84], and promoter methylation in ulcerative colitis [134].

TUSC3 is localized in the endoplasmic reticulum and encodes a subunit of endoplasmic reticulum-bound oligosaccharyltransferase, which plays a crucial role in protein N-glycosylation [76,101-103]. Recently, Vaňhara *et al.* identified the essential role of TUSC3 as tumor suppressor gene in ovarian cancer in addition to the observations suggested its function in N-glycosylation [76]. Mohorko *et al.* suggested crucial role of TUSC3 subunits which is necessary for N-glycosylation in the brain development [93].

It is also known as an Mg^{2+} transporter involved in vertebrate plasma membrane magnesium transport. Knockdown of *Tusc3* gene expression was found to lower the total and free intracellular Mg^{2+} concentration in a human cell line, as well as to induce developmental arrest in zebra fish, which was rescued by supplementation with excess Mg^{2+} [59]. Intracellular magnesium is abundant, highly regulated and plays important roles in biochemical functions, morphological and cytological changes. Significant increase of the androgenic enzyme activity was observed by magnesium administration in the rat testis, and it has important role on spermatogeneisis [61, 62]. Wong *et al* reported the role of magnesium with other cations as playing an important role in spermatogenesis and fertility [62]. Moreover, Chandra *et al*. observations supported the idea by studying the effect of dietary magnesium on testicular histology, which has beneficial effect on male gonadal system [61].

Recent studies have shown that mutation in *Tusc3* causes several types of mental retardation, especially the non-syndromic type [90-95]. Garashabi *et al.* investigated three mentally retarded

patients; they identified a novel nonsense mutation in the second exon of *Tusc3* gene leading to mental retardation [90]. Garashabi *et al.* suggested that *Tusc3* gene defects of glycosylation resulted to nonsyndromic mental retardation [91].

Although TUSC3 is known to have multiple functions, and is related to tumor suppression in prostate cancer and mental retardation, its importance in spermatogenesis is totally unknown. In this study, therefore, I performed expression analysis of *Tusc3* and its gene product, and elucidated its function on spermatogenesis.

4.3 Materials and Methods

DNA microarray analysis

Sprague-Dawley (SD) rat organs were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). TRIzol reagent (Life Technologies, Carlsbad, CA) was used to prepare total RNA from the testis of 2-week-old and 7-week-old rats according to the manufacturer's instructions. Custom DNA microarray analysis was performed using the service provided by Filgen, Inc. (Nagoya, Japan). In brief, first strand cDNA was synthesized using ArrayScript™Reverse Transcriptase from 1 μg of individual total RNA as a template. After the second strand cDNA synthesis, biotin-labeled antisense RNA (aRNA) was synthesized using T7 enzyme using the cDNA as a template. The biotin-labeled aRNA was fragmented and hybridized with CodeLink™ Rat Whole Genome Bioarray (Applied Microarrays, Inc., Tempe, AZ) in which 33,849 probes are loaded. Hybridized slides were washed and incubated with Cy™5-streptavidin working solution (GE Healthcare, Buckinghamshire, UK) then scanned using GenePix® 4000B (Molecular Devices, Inc., Sunnyvale, CA). Scanned image files were analyzed using CodeLink Expression Analysis v5.0 software (Applied Microarrays, Inc.).

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared from testes at various developmental stages of 2-, 3-, 7-, 9- and 15-week-old, prostate at 2-, 3-, 9-week-old, and from individual organs of 9-week-old male and female rats. A first-strand cDNA synthesis kit including Super Script III Reverse Transcriptase was used to synthesize cDNA from 1 μg RNA (Life Technologies). PCR amplified 515 bp of *Tusc3* (accession no. NM_001004212) within the open reading frame using One Taq DNA polymerase (New England Biolabs, Ipswich, MA) with the following conditions: 94°C for 5 min,

then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by an extension at 72°C for 10 min. PCR amplification primers were as follows: rat *Tusc3* sense primer, 5'-CCGCAGGTTGCGCTACCTGTCTAC-3' and antisense primer, 5'-TCCTGTCGGCAATCCATTTTGCTAGCTG-3'. *Tusc3* cDNA was cloned into the pGEM-T Easy vector and sequenced to confirm the precise amplification before being used for further analysis. Mammalian *Gapdh* primers 5'- AGTCGGAGTGAACGGATTTGG -3', and antisense primer 5'-AGTTGTCATGGATGACCTTGG -3' were used for PCR amplification as internal control.

Northern blotting

For Northern blotting, electrophoresis of 5 μg RNA was performed in a formaldehyde-containing denaturing gel that was blotted on a Hybond N^+ nylon membrane (GE Healthcare). The PCR-amplified *Tusc3* cDNA probe was labeled with [α-^{32}P] dCTP using a random primer labeling system (GE Healthcare). After hybridizing the membrane with the probe, it was washed at 65°C for 20 min with buffer containing 1 × saline sodium citrate (SSC) and 0.1% SDS. Radioactivity was measured using a FLA-5000 fluorescent image analyzer (GE Healthcare). The membrane was rehybridized with rat *Gapdh* cDNA as an internal control to determine the RNA integrity and quantity.

In situ hybridization

A DNA probe was synthesized by asymmetric PCR involving 40 cycles using the individual PCR primer, 10 ng of *Tusc3* cloned plasmid DNA as a template and the DIG DNA Labeling Mix (Roche, Basel, Switzerland). The following conditions were applied : 94°C for 5 min, then 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by an extension at 72°C

for 10 min. Amplified cDNA was purified by the MonoFas DNA Purification Kit I (GL Sciences Inc., Torrance, CA). Five-micrometer sections of 3- and 8-week-old rat testis and prostate were hybridized with sense and antisense probes, and the signals were developed using a DIG Nucleic Acid Detection Kit (Roche) following the manufacturer's instructions. The signals were detected using a DAB chromogen substrate kit (Dako, Carpinteria, CA), and the sections were counterstained with hematoxylin.

Western blotting and immunohistochemistry

Complete Lysis-M (Roche Applied Science, Tokyo) was used to prepare rat tissue proteins, then 25 μg of each protein sample was electrophoresed on a sodium dodecyl sulphate 12% polyacrylamide gel (SDS-PAGE) and transferred to a Hybond C membrane (GE Healthcare). TUSC3 antisera (SAB4503183, Sigma-Aldrich, Saint Louis, MO) (1:1,000) was used to hybridize the membrane, which was then incubated with HRP-conjugated goat anti-rabbit IgG (Cosmo Bio, Tokyo) (1:10,000) as a secondary antibody. The Immunoblot Western Chemiluminescent HRP Substrate (Millipore, Tokyo) detected bound antibody. Rabbit polyclonal anti-GAPDH (Cosmo Bio) was used as a control primary antibody (1:2,000).

The same primary antisera (1:100) used in Western blotting was hybridized to the sections used in ISH. Signals were detected using a Histofine DAB substrate kit (Nichirei, Tokyo) following the manufacturer's instructions.

4.4 Results

DNA microarray, RT-PCR, Northern blotting, and Western blotting

We used Rat Whole Genome DNA microarray to screen the genes that were expressed specifically during the sexual maturation of rat testis. Loaded probe number was 33,849, and we focused on the genes, in which the expression was more than twice difference. Among the loaded probes, 4,322 candidates were up-regulated, and 5,626 were down-regulated. From the up-regulated genes, we chose *Tusc3* gene as a tumor suppressor candidate, and the expression was 2.4 fold increased from 2- to 7-week-old rat testis according to the DNA microarray analysis. *Tusc3* gene expression was determined by RT-PCR and a 515 bp specific band was observed, and by Northern blotting, slightly lower than 18S rRNA-sized band, which agreed with the mRNA size of 1,514 bases was observed. Its expression was lower in 2-week-old testis, and then it was up-regulated by the age of 3 weeks and was maintained to 15 weeks during testicular development. Strong expression of *Tusc3* was detected in maturing rat testis, while lower expression was observed in the cerebrum and ovary of 9-week-old rats by RT-PCR and Northern blot analysis (Fig. 4-1A, B). TUSC3 protein expression was determined by Western blotting and shown to be weak in the testis of 2-week-old rats, increasing after 3 weeks of age and maintained thereafter. Protein size was approximately 40 kDa in the developing testis, cerebrum, and ovary, which corresponds to the reported database size of 347 amino acids (Fig. 4-1C).

Gene expression was also determined for prostate developmental stages. *Tusc3* gene expression analysis showed the presence of a specific band, similar to testis expression, in RT-PCR and Northern blotting (Fig. 4-2A, B). Expression was strong and at the same level in all

stages tested during prostate development and the intensity was relative to the testis expression. The relative expression intensity in the prostate of individual stages was 98 to 105% compared to that of the testis 9-weeks, according to the densitometric analysis. TUSC3 protein expression was shown by Western blotting to be the same level during prostate developmental stages, and confirmed the size of 40 kDa (Fig. 4-2C).

ISH and immunohistochemistry

Gene expression in the rat testis sections was determined by ISH. *Tusc3* gene expression was evident in the spermatocytes of 3-week-old rat testis (Fig. 4-3A), and was maintained in 8-week-old spermatocytes. The expression was also observed in interstitial cells such as Leydig cells (Fig. 4-3C, D, and E). Signals from sense probes were negative in both 3- and 8-week-old samples (Fig. 4-3B and F).

Immunohistochemistry revealed that TUSC3 protein expression in spermatocytes mirrored *Tusc3* gene expression by occurring in 3-week-old testis (Fig. 4-4A) and continuing to 8 weeks of age in spermatocytes and Leydig cells (Fig. 4-4C, D, and E). Preimmune sera signals were negative in both 3- and 8-week-old samples (Fig. 4-4B and F).

Gene and protein expression in the rat prostate sections was determined by ISH and immunohistochemistry. *Tusc3* gene expression occurred in the epithelia of 3- and 8-week-old rat prostate (Fig. 4-5A and C), while sense probes were negative (Fig. 4-5B and D) by ISH. Immunohistochemistry showed similar expression to ISH, and was detected in prostate epithelia at 3 and 8 weeks of age (Fig. 4-5E and G). Signals with preimmune sera were negative (Fig. 4-5 F and H).

4.5 Discussion

Tumor suppressor genes are interesting targets for exploring the molecular mechanisms of cell division and differentiation, and they have been researched for their roles in cancer diagnosis and treatment. These genes have been screened and characterized into two classes, class I and class II, and their products have been found to be closely related to carcinogenesis and metastasis [135]. Class I suppressor gene products play direct roles in the cell cycle checkpoint, protein degradation, tumor cell adhesion, and DNA repair [136-138]. For example, E-cadherin is associated with adherence at the calcium-dependent epithelial cell–cell junction, so loss of its function may lead to cancer metastasis [137]. Class II suppressor gene products play similar roles to class I cancer suppressor gene products in cancer suppression, but class II suppressor genes are not usually mutated, and their expression is regulated by some of the class I suppressor gene products. Some of the class I suppressor gene products coordinately regulate the expression of several class II suppressor genes as transcription regulators. Retinoblastoma (RB), Wilms' tumor (WT1), and p53 are known transcription factors which control downstream class II suppressor gene expression [135]. The human HRev107 family is known to be class II suppressor candidate, and expression of one of its members, retinoid-inducible gene 1 (*RIG1*), is regulated by p53 [139]. HRev107 expression has been observed in round spermatids but is lost in testicular germ cell tumor [140]. Rat lecithin-retinol acyltransferase-like protein 1 (RLP-1) is coded by the *hrasls5* gene and is known to be a homolog of human HRev107 [57, 141]. However, the importance of RLP-1 expression in the developing testis with regard to tumor suppression and transacylation activity is not yet understood. These findings indicate the possibility of enzyme activation during tumor suppression. Accordingly, we have screened genes related to tumor suppression and have induced additional functions such as enzyme

activation. DNA microarray analysis is a valuable method for screening the total compliment of spermatogenesis-related genes because of its high throughput. We classified genes screened by this method according to their expected functions.

In this study, I chose a tumor-suppressor candidate, the *Tusc3* gene, because its expression was up-regulated during sexual maturation in the testis according to DNA microarray analysis. *Tusc3* codes a subunit of oligosaccharyltransferase, and the product is expected to be an Mg^{2+} transporter.

Many vertebrate plasma membrane transporters have been reported to transport divalent cations across the plasma membrane. Of these, TUSC3, MagT1, and NIPA2 are known to be deeply involved in Mg^{2+} transport [59, 60]. The modification and regulation of the Mg^{2+} concentration in the cytoplasm of the developing prostate and testis have not been clarified, even though Mg^{2+} is important for the significant increase in androgenic enzyme activity in human and rat testis [61, 62]. The TUSC3 protein has been shown to modulate intracellular Mg^{2+} concentration in both a human cell line and in zebra fish, and it is also related to embryonic development [59]. TUSC3 expression in Leydig cells, as well as in earlier spermatogenic cells and the prostate epithelium, suggests that an increase in androgenic enzyme activity and the modulation of serum testosterone concentration leads to spermatogenesis. Magnesium in the prostate may assist with enzyme activation, and a reduction in magnesium levels may be associated with prostatitis and other disorders [96]. Thus, the magnesium concentration in seminal plasma is proposed to be a valuable marker for prostatitis. Consistent with reported data, we detected similar levels of TUSC3 expression in the epithelia of infant to mature prostate. It is conceivable that life-long expression of TUSC3 in the prostate may be involved in the

magnesium transport necessary to activate related enzymes according to their correct prostate function.

In the present study, *Tusc3* expression in Leydig cells and in the developing testis was observed, where it was stronger in spermatocytes compared with spermatids. Faint expression was detected in 2-week-old rat testis, after which it increased from 3 weeks of age. Leptotene spermatocytes appear in the seminiferous tubules after 2 weeks of age, while pachytene spermatocytes appear at 3 weeks of age [35]. Our histological analysis by *in situ* hybridization and the immunohistochemistry of spermatogenic cells indicate that most of the TUSC3 expression is concluded in pachytene spermatocytes.

In future, it will be interesting to determine the molecular mechanism of TUSC3 in pachytene spermatocyte meiosis, differentiation and development, and prostate function. Doing so will elucidate the common mechanism of TUSC3 in spermatogenesis, cancer suppression, and mental retardation, and we expect that the mechanism of TUSC3 is related to its roles in Mg^{2+} transport and enzyme activity.

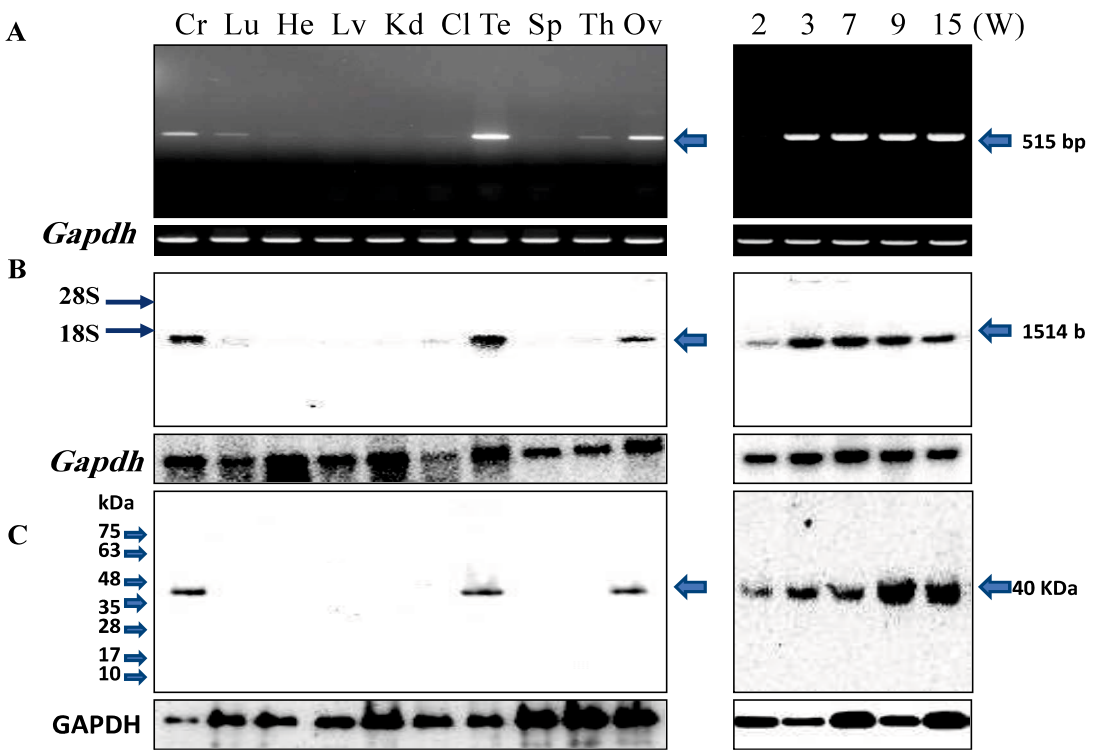

Fig. 4-1. RT-PCR and Northern blot analysis of rat *Tusc3* transcript and TUSC3 protein in the testis

A, *Tusc3* RT-PCR transcript analysis of organs of 9-week-old rats (Cr; cerebrum, Lu; lung, He; heart, Lv; liver, Kd; kidney, Cl; colon, Te; testis, Sp; spleen, Th; thymus, Ov; ovary), and 2-, 3-, 7-, 9-, and 15-week (W)-old rat testis. Arrows indicate 515 bp amplified fragments. Rat *Gapdh* amplifications are indicated at the bottom. **B**, *Tusc3* transcript analyzed by Northern blotting. Arrows on the left indicate positions of ribosomal RNA (28S rRNA and 18S rRNA). Arrows on the right indicate the position of *Tusc3* mRNA (1,514 bases). Rat *Gapdh* expression is indicated at the bottom. **C**, TUSC3 protein analysis by Western blotting. Arrows on the left indicate positions of protein standards. Arrows on the right indicate TUSC3 signal position (40 kDa). Anti GAPDH antibody signal is indicated at the bottom.

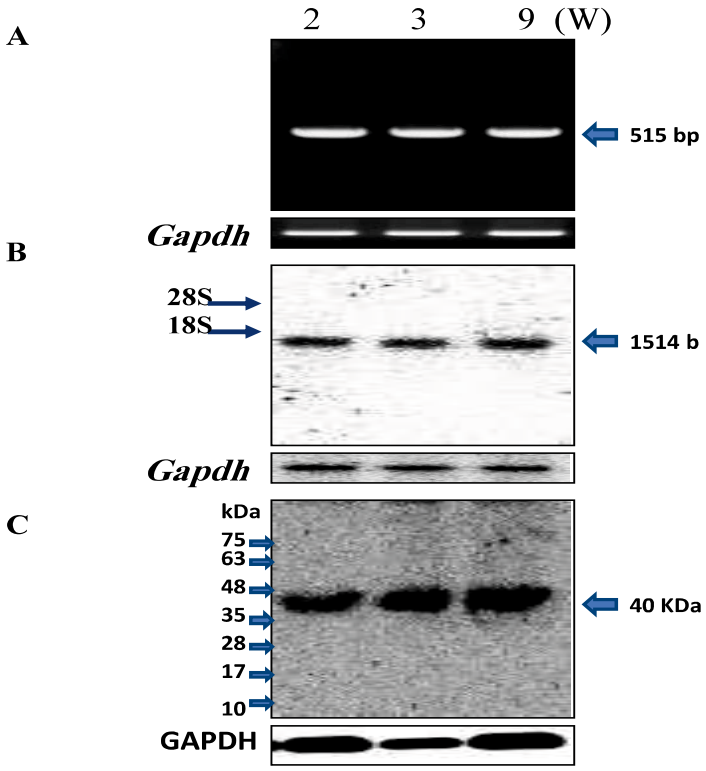

Fig. 4-2. RT-PCR and Northern blot analysis of rat *Tusc3* transcript and TUSC3 protein in the prostate

A, *Tusc3* RT-PCR transcript analysis of 2, 3, and 9-week-old rat prostate. An arrow indicates 515 bp amplified fragments. Rat *Gapdh* amplifications are indicated at the bottom. **B**, *Tusc3* transcript analyzed by Northern blotting. An arrows on the left indicate positions of ribosomal RNA (28S rRNA and 18S rRNA). An arrow on the right indicates *Tusc3* mRNA signal. Rat *Gapdh* expression is indicated at the bottom. **C**, TUSC3 protein analysis by Western blotting. Arrows on the left indicate positions of protein standards. An arrow on the right indicates TUSC3 signal position (40 kDa). Anti GAPDH antibody signal is indicated at the bottom.

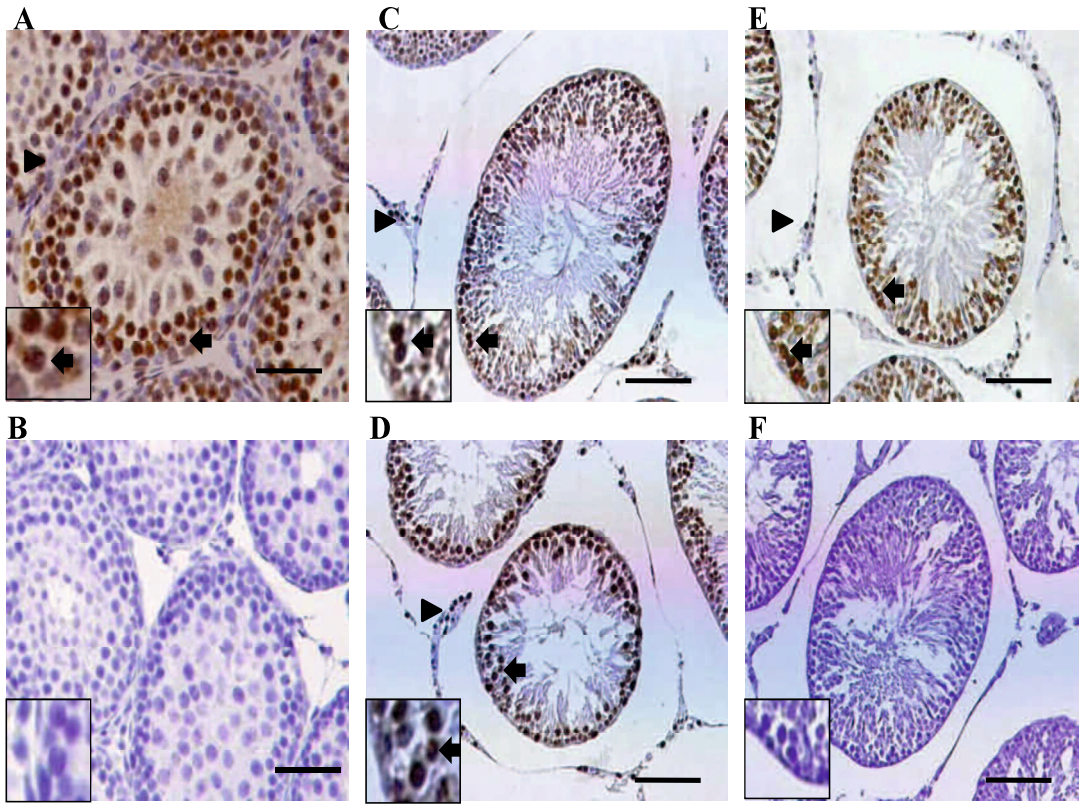

Fig. 4-3. Cellular localization of rat *Tusc3* transcript in 3- and 8-week-old rat testis

In situ hybridization using the *Tusc3* probe and 5-μm-thick sections of 3-week-old rat testis seminiferous tubules (A) and 8-week-old rat testis at various stages of seminiferous tubule development (C–E). Sense probe used as a negative control for 3- and 8-week-old samples, respectively (B and F). The tubules of 8-week-old rat testis were expected to be (C) stages I to IV, (D) stages VII to VIII, and (E) stages IX to XI. Signals (brown) in the spermatocytes (arrows) and Leydig cells (arrowhead) can be observed. High magnification pictures from individual tubules are indicated in boxes. Sections were counter stained with hematoxylin. Scale bar, 100 μm.

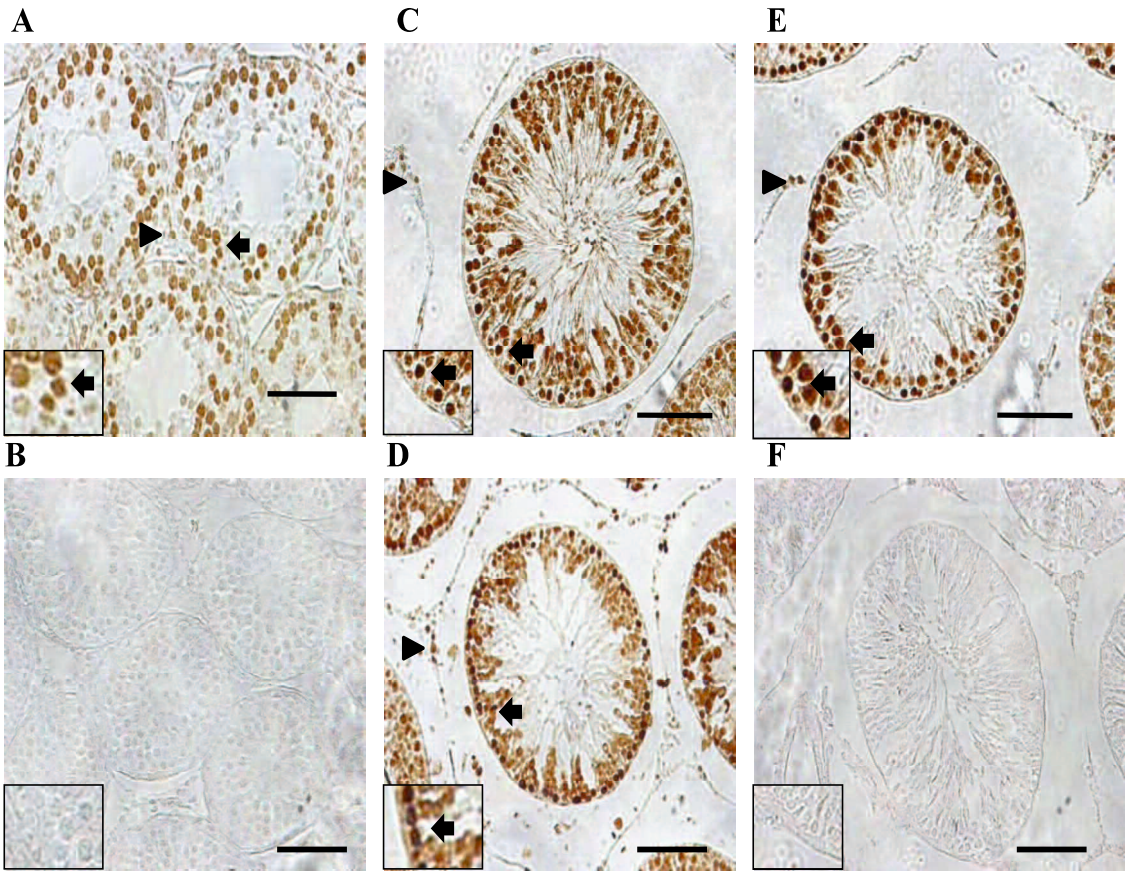

Fig. 4-4. Cellular localization of rat TUSC3 protein in 3- and 8-week-old rat testis

Immunohistochemistry using rat TUSC3 antisera and 5-μm-thick sections of 3-week-old rat testis seminiferous tubules (A) and 8-week-old rat testis at various stages of seminiferous tubule development (C–E). Pre-immune serum was used as a negative control for 3- and 8-week-old samples, respectively (B and F). The tubules of 8-week-old rat testis were expected to be (C) stages I to IV, (D) stages VII to VIII, and (E) stages IX to XI. Signals (brown) in the spermatocytes (arrows) and Leydig cells (arrowhead) can be observed. High magnification pictures from individual tubules are indicated in boxes. Scale bar, 100 μm.

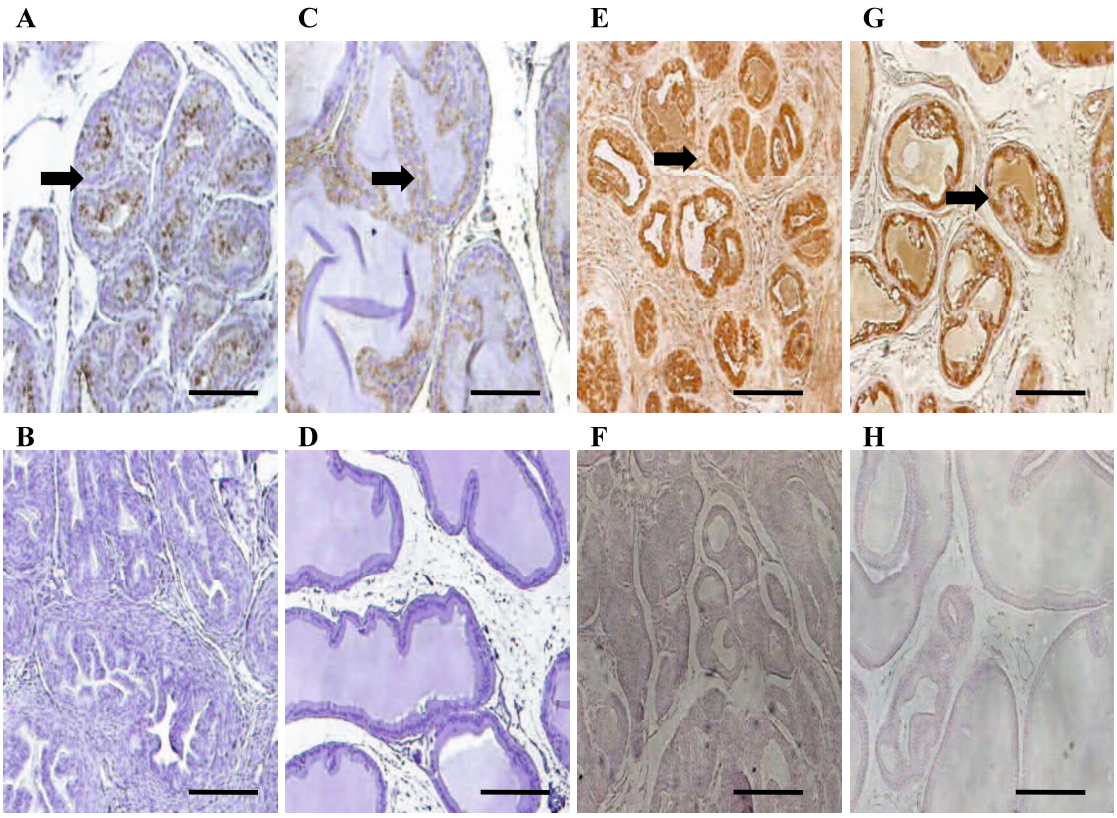

Fig.4- 5. Cellular localization of rat *Tusc3* mRNA and TUSC3 protein in 8-week-old rat prostate

In situ hybridization using the *Tusc3* probe and 5-µm-thick sections of 3- and 8-week-old rat prostate (A and C), and hybridized with a sense probe as a negative control (B and D). Signals (brown) in the epithelium (arrows) can be observed. Sections were counter stained with hematoxylin. Immunohistochemistry using rat TUSC3 antisera on 5-µm-thick sections of 3- and 8-week-old rat prostate (E and G), and using pre-immune serum as a negative control (F and H). Signals (brown) in the epithelium (arrows) can be observed. Scale bar, 100 µm.

Chapter V

Summary and Conclusions

The aim of this study is to characterize the genes which are involved in spermatogenesis, analyze their expression, and build a solid base to elucidate their different functions. This may lead to understand the total process of the spermatogenesis from genetic background. Results of screening during spermatogenesis revealed several genes which express during maturation; which are interesting targets because during this process dynamic cell division and morphological changes takes place. Many of these genes are commonly expressed in somatic cells and some of them are related to certain diseases such as cancer. For this purpose I have screened genes specifically expressed in developing testis and characterized their expression according to their expected functions.

In the present study I have screened, identified, and characterized many specific genes from maturing rat testes by differential display (DD). I chose some candidates which are up-regulated in the maturing rat testes. While the functions of some of these have been determined by comparing DNA sequences in the database, many functions remain unknown.

In Chapter I, I have summarized the background of the study. In Chapter II, I focused on a candidate gene *LOC290876,* of unknown biological function. According to ENSEMBL survey I found this gene to contain three EDG-8 motifs. The expression was testis specific and increased at week 7 and continued for 15 weeks. PCR analysis revealed two gene transcript isoforms with similar expression level, which confirmed later by Northern blotting analysis. I identified two splicing variants of *LOC290876.*The deduced amino acid sequences of the two isoforms revealed differences in carboxyl terminal sequences. The major expression of the gene and protein in the testes was in the spermatocytes and the protein expression was localized to the nucleus. In conclusion I suggested that the *LOC290876*- encoded gene product is not involved in

sphingosine signaling, but has crucial roles in the nucleus during the processes of spermatocyte maturation and meiosis producing spermatids.

In Chapter III, I studied the expression of the T-cell acute-lymphocytic leukemia (*Tal-1*) gene, suggested CT antigen candidate, in maturing rat testes. TAL-1 has a basic helix-loop-helix (bHLH) domain and can be a positive regulator of several downstream genes expressed in the bone marrow in relation to leukemia. According to the known function of TAL-1, which plays an important role in angiogenesis and hematopoietic differentiation such as bone marrow erythropoiesis during embryogenesis, as well as in T cell acute lymphocytic leukemia (T-ALL) development. I expected additional function of the gene product on spermatogenesis. Strong expression was detected in the normal maturing rat testes by Northern blotting. Western blotting revealed the protein size to be 34 kDa. Since the expected protein molecular size which reported in sequence database as 22.7 kDa, I analyzed the upstream sequence of the proposed methionine codon, considering the existence of longer ORF. I found that rat testis (Rat-TE) DNA sequence was different from the data base (Rat DB; accession no. NM_001107958). Accordingly, rat *Tal-1* ORF was determined to be 329 amino acids. Protien expression was observed wide-spread in the spermatocytes , spermatids and spermatogonia by immunohistochemistry. Gene expression of *Tal-1* regulatory genes, *NKX3.1*, was negatively correlated with *Tal-1* expression. Human *Tal-1* expression in the maturing testis and bone marrow was observed, which suggest *Tal-1* as a novel cancer-testis antigen candidate. In conclusion, TAL-1 may be involved in cell division, morphological changes, and the development of spermatogenic cells in the normal rat testes.

Finally, in Chapter IV, I utilized DNA microarray considering further high throughput screening of spermatogenesis related genes. I focused on one candidate, tumor suppressor candidate gene 3 (*Tusc3*). The expression was up-regulated in developing rat testis. It is known

that *Tusc3* is involved in several biological functions such as magnesium transporter, related to tumor suppression, mental retardation, and expected role in protein N-glycosylation, but the function in spermatogenesis is totally unknown. RT-PCR and Northern blotting showed *Tusc3* gene expression was up-reglated in the normal maturing testes and prostate and other organs such as cerebrum and ovary. TUSC3 protein expression was detected in the same organs with a size of around 40 kDa. *In situ* hybridization and immunohistochemistry showed that mRNA and protein localization was in the spermatocytes and Leydig cells, as well as prostate epithelial cells. These findings, suggest the involvement of TUSC3 in spermatogenesis in the rat testis to induce sperm differentiation and maturation and play important role in prostate development and tumor suppression.

In future work, it will be interesting to determine the precise physiological functions of each candidate to understand the molecular basis to unravel their role in relation to many diseases such as tumor development and suppression in addition to better understanding of spermatogenesis. Gene knockdown experiment in the developing testis may lead to further understanding for the individual gene functions.

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List of publications

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Khalid AM, Asano A, Hosaka YZ, Ohta M, Ohyama K, and Yamano Y. 2012. Rat stem-cell leukemia gene expression increased during testis maturation. *Biosci. Biotechnol. Biochem*. (IF=1.269), **76:** 2118–2123……..……..….**Chapter III**

Khalid AM, Asano A, Hosaka YZ, Takeuchi T, and Yamano Y. 2013. Tumor Suppressor Candidate TUSC3 Expression during Testis Maturation. *Biosci. Biotechnol. Biochem*. Manuscript in press……..……...……..**Chapter IV**

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