REGULATION OF SPERMATOGENESIS:

Differentiation of GFP-labeled Stem Cells and the Function of Cytoplasmic Bridges

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LIST OF ORIGINAL COMMUNICATIONS

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I. Ventelä S, Okabe M, Tanaka H, Nishimune Y, Toppari J, Parvinen M (2000) Expression of green fluorescent protein under beta-actin promoter in living spermatogenic cells of the mouse: stage-specific regulation by FSH. Int J Androl 23:236-242

II. Ventelä S, Mulari M, Okabe M, Tanaka H, Nishimune Y, Toppari J, Parvinen M (2000) Regulation of acrosome formation in mice expressing green fluorescent protein as a marker. Tissue Cell 32:501-507

III. Egydio de Carvalho C, Tanaka H, Iguchi N, Ventelä S, Nojima H, Nishimune Y (2002) Molecular cloning and characterization of a complementary DNA encoding sperm tail protein SHIPPO 1. Biol Reprod 66:785-795

IV. Ventelä S, Ohta H, Parvinen M, Nishimune Y (2002) Development of the stages of the cycle in mouse seminiferous epithelium after transplantation of green fluorescent protein-labeled spermatogonial stem cells. Biol Reprod 66:1422-1429

V. Ventelä S, Toppari J, Parvinen M (2002) Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: Mechanisms of haploid gene product sharing. *Submitted.*

LIST OF ABBREVIATIONS

Acr	Acrosin
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ES	Embryonic stem cells
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
LH	Luteinizing hormone

ABSTRACT

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REGULATION OF SPERMATOGENESIS: Differentiation of GFP-labeled stem cells and the function of cytoplasmic bridges
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During spermatogenesis, different genes are expressed in a strictly coordinated fashion providing an excellent model to study cell differentiation. Recent identification of testis specific genes and the development of green fluorescence protein (GFP) transgene technology and an in vivo system for studying the differentiation of transplanted male germ cells in infertile testis has opened new possibilities for studying the male germ cell differentiation at molecular level. We have employed these techniques in combination with transillumination based stage recognition (Parvinen and Vanha-Perttula, 1972) and squash preparation techniques (Parvinen and Hecht, 1981) to study the regulation of male germ cell differentiation.

By using transgenic mice expressing enhanced-(E)GFP as a marker we have studied the expression and hormonal regulation of beta-actin and acrosin proteins in the developmentally different living male germ cells. Beta-actin was demonstrated in all male germ cells, whereas acrosin was expressed only in late meiotic and in postmeiotic cells. Follicle stimulating hormone stimulated β-actin-EGFP expression at stages I-VI and enhanced the formation of microtubules in spermatids and this way reduced the size of the acrosomic system. When EGFP expressing spermatogonial stem cells were transplanted into infertile mouse testis differentiation and the synchronized development of male germ cells could be observed during six months observation time. Each colony developed independently and maintained typical stage-dependent cell associations. Furthermore, if more than two colonies were fused, each of them was adjusted to one stage and synchronized. By studying living spermatids we were able to demonstrate novel functions for Golgi complex and chromatoid body in material sharing between neighbor spermatids. Immunosytochemical analyses revealed a transport of haploid cell specific proteins in spermatids (TRA54 and Shippo1) and through the intercellular bridges (TRA54). Cytoskeleton inhibitor (nocodazole) demonstrated the importance of microtubules in material sharing between spermatids and in preserving the integrity of the chromatoid body. Golgi complex inhibitor, brefeldin A, revealed the great importance of Golgi complex i) in acrosomic system formation ii) TRA54 translation and in iii) granule trafficking between spermatids.

Key words: testis, spermatogenesis, green fluorescent protein, beta-actin, follicle stimulating hormone, acrosin, transplantation, haploid, microtubules, Golgi complex, stem cells

INTRODUCTION

The roots of stem cell research go back to year 1924, when Spemann and Mangold demonstrated that embryos of ri urus contain totipotent cells which are capable to differentiate to nerve-, muscle- or epithelial cells, depending on the culturing conditions. The lack of stem cells or their inability to differentiate is problematic when it comes to incurable disease where the internal regeneration of the cells or external treatments are not curative (certain infertilities, disorders in nervous system, heart disease). In these situations stem cell transplantation has been proposed to be therapeutically promising. Although, the studies of stem cells have obvious clinical opportunities they also give us new methods to study the regulation and mechanisms needed in cell differentiation. Classically, mammalian stem cells have been studied in the tissues such as blood and epidermis, where the differentiated cells do not divide and have short life-span. However, the positions of stem cells is known only approximately in many tissues making it very problematic to study the microenvironments, known as niches, regulating the stem cell fate (Watt and Hogan, 2000). The simple morphology and accessibility of the testis combined with in vivo assay system where spermatogonial stem cells are transplanted into infertile testis (Brinster and Zimmermann, 1994) offer vast experimental advantages to study stem cell niches regulating spermatogenesis (Spradling et al., 2001). Moreover, recent findings and development in transgene technology (characterization and isolation of green fluorescent protein) and in living cell studies (digital imaging techniques) have opened new insights to study the phenomenon happening in living cells and the regulation of cell differentiation.

We have employed these techniques to transillumination based stage recognition (Parvinen and Vanha-Perttula, 1972) and squash preparation techniques (Parvinen and Hecht, 1981) to study the regulation of male germ cell differentiation in green fluorescent protein (GFP) transgenic mice. We have also examined transplanted spermatogenig cells (Brinster and Zimmermann, 1994) and the regulation of haploid cell specific gene expression.

REVIEW OF THE LITTERATURE

1. STEM CELL DIFFERENTIATION

1.1 Background of the studies of stem cells

Stem cells have recently increased in interest because of demonstration of Thomson et al. (1998) that pluripotent human embryonic stem cells maintain their differentiation potential in vitro even after long period of culturing. This result aroused wide scientific and ethical discussion about the risks and possibilities of stem cells.

There exist two types of stem cells: pluripotent stem cells giving rise to all types of cells and tissue specific stem cells which contain more restricted differentiation ability. Embryonic stem (ES) contain pluripotent nature and are responsible for organogenesis, whereas tissue specific (adult) stem cells work as a supply of cells needed in different tissues during regeneration or healing after trauma. The lack of stem cells or their inability to differentiate is problematic when it comes to incurable disease where the internal regeneration of the cells or external treatments are not curative (certain infertilities, disorders in nervous system, heart disease). In these situations stem cell transplantation had been proposed to be therapeutically promising.

1.2 Applications of stem cells

Previously it was believed that transplanted embryonic stem (ES) cells would repair at cell level the damage of the devastating disorders, such as Parkinson's disease, diabetes, and heart diseases. However, recently it has been demonstrated that when undifferentiated ES cells are transplanted they tend to form teratomas in the recipient individual (Thomson et al., 1998). This has raised a hypothesis that ES cells should be reliably differentiated into the appropriate cell type in the presence of desired local regulators (transcription factors, hormones) in culture conditions. According to recent findings this might be the situation, at least in the case of Parkinson's disease (Kim et al., 2002). Kim et al (2002) cultured ES cells in the presence of Nurr1 (transcription factor, which is needed in the differentiation of dopamin neurons) before the transplantation of ES cells into rats with symptoms of Parkinson's disease. The transplantation of modified ES cells led to the recovery of Parkinson's disease in rats. This result demonstrates the potential of stem cells and increases the importance of understanding the role of local regulators during the stem cell differentiation.

Previously also many achievements have been gained in the studies when so-called tissue specific stem are used as a transplant. With this method it has been possible to treat animal models containing malfunctions in liver (Braun et al., 2000), heart (Orlic et al., 2001), male infertility (Brinster and Zimmermann, 1994) and bone marrow (Jantunen et al., 2001). Although these studies open obvious

clinical possibilities they also give us new methods to study the regulation and mechanisms needed in basic studies of cell differentiation.

2. MALE GERM CELL DIFFERENTIATION

2.1 Spermatogenesis

Male germ cell differentiation is a highly regulated, complex process that takes place in the seminiferous tubules of testis. Spermatogenesis can be subdivided into 3 main phases: (i) spermatogonial proliferation, (ii) meiosis of spermatocytes and (iii) spermiogenesis, a morphological processes convertin haploid spermatids to spermatozoa (Leblond and Clermont, 1952). In a cross section of the seminiferous tubule the spermatogenic stem cells, other premeiotic germ cells and somatic Sertoli cells are situated on the basal lamina of the seminiferous tubule. The Sertoli cells surround completely the germ cells beyond the onset of meiosis. The next layer is formed by spermatocytes while haploid spermatids and elongated spermatids are situated in the adluminal compartment (Fig. 1).

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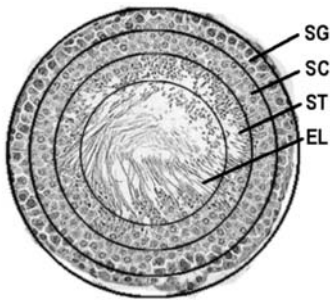
Figure 1. Histological cross section of seminiferous tubule (Figure 1.avi). In basal compartment locate the spermatogonial stem- and progenitor cells (SG). Spermatocytes (SC) and haploid cells: round spermatids (ST) and elongated spermatids (EL) are situated in the adluminal compartment.

During spermatogenesis male germ cells undergo a complex differentiation where morphological alterations lead to the formation of differentiated sperm. Spermatogenesis starts when a spermatogenic stem cell (called A_s spermatogonia) gives rise two daughter cells after initial division (de Rooij, 2001). One of these cells remains as a stem cell while the other enters spermatogenesis as a differentiating spermatogonia [called A_{pr} (paired) and further A_{al} (aligned) spermatogonia]. In stages VII-VIII nearly all A_{al} spermatogonia differentiate into A1 spermatogonia which divide mitotically six times (Clermont, 1962) giving rise A2-A4, intermediate, and B-type spermatogonia which can be identified based on morphological criteria (Russell et al., 1990). A characteristic feature of spermatogenesis is that after mitotic and meiotic divisions the dividing germ cells fail to complete the cytokinesis resulting in formation of cytoplasmic bridges that interconnect a large number of cells (Burgos and Fawcett, 1955; Fawcett et al., 1959). Kinetic analyses reveal that hundreds or even thousands of cells theoretically may be connected by bridges at the completion of spermatogenesis (Dym and Fawcett, 1971). After the final mitotic division of type B-spermatogonia preleptotene spermatocytes are formed, which initiate meiosis and give rise to leptotene and zygotene spermatocytes. These cells differentiate into pachytene and diplotene spermatocytes followed by meiotic divisions and

formation of haploid step 1 spermatids (spermiogenesis starts). Haploid spermatids are morphologically classified into 16 steps in mouse and 19 in rats (Russell et al., 1990). Based on the morphological criteria of spermatids, spermatogenesis can be subdivided into different cell associations, also called stages.

2.2 The cycle and wave of the seminiferous epithelium

Inside the seminiferous tubules, developmentally different male germ cells form defined cell associations which follow each other in sequential order. These defined cell associations inside the seminiferous tubules can be distinguished into twelve (in mouse; Oakberg 1956) or fourteen (in rats; Leblond and Clermont, 1952) different stages of the seminiferous epithelial cycle (Fig. 2). Each stage contains male germ cells of defined phases of differentiation and can be accurately identified by the morphological features of the acrosome and of the nucleus of developing spermatids. The duration of the cycle is constant: stage I development to stage XIV is 12.9 d in the Sprague Dawley rat and 8.6 d in the mouse (stage I to stage XII).
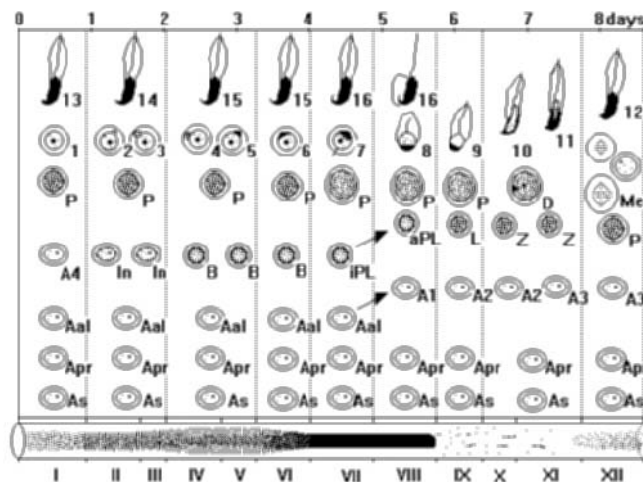
Figure 2. The seminiferous epithelial cycle in the mouse, as described by de Rooij (1998). Each stage of the cycle, denoted by Roman numerals, contain defined cellular associations, which give the seminiferous epithelium its characteristic appearance under transillumination microscope (Parvinen and Hecht, 1981).

Different segments of the seminiferous epithelial wave absorb light in a different way making it possible to identify the stages in freshly isolated seminiferous tubules under a transillumination stereomicroscope (Parvinen and Vanha-Perttula, 1972; Parvinen and Hecht, 1981). Increased light absorption is associated with nucleoprotein changes and progressive chromatin condensation of haploid germ cells. Stages II-VI can be identified thorough the dark spots that are spermatid bundles associated with the Sertoli cells. At stages VII-VIII a dark absorption zone at the lumen of seminiferous tubule is seen. The formation of a pale absorption zone at stages IX-XII (in rat) and IX-X (in mouse) is formed after spermiation. Following stages XIII-I (in rat) and XI-I (in mouse) form a weak spots in the tubular center during the replacement of histones with protamines.

2.3 Hormonal control of spermatogenesis

The most important somatic cells of the testis are Sertoli, peritubular myoid and Leydig cells. These cells are responsible for the hormonal regulation of the spermatogenesis. Gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are secreted from the cells in the anterior pituitary. LH binds to the receptors of Leydig cells which are situated in the interstitial tissue. LH- stimulation of Leydig cells causes the production of testosterone, which is the major hormone supporting adult spermatogenesis, both in qualitative and quantitative manner. The action of testosterone is mediated through nuclear androgen receptors which are localized in Sertoli, peritubular myoid and Leydig cells (Bremner et al., 1994).

In immature testis follicle-stimulating hormone (FSH) is essential for initiation and maintenance of spermatogenesis. In adult testis FSH supports the function of Sertoli cells, which in turn support many aspects of sperm cell maturation. FSH-receptors are most abundant in Sertoli cells of rat at stages XIII-I of the seminiferous epithelial cycle (Kangasniemi et al., 1990) where, for instance, spermatogonia differentiation is initiated. Although the precise functions of FSH at cellular level have remained elusive (Simoni et al., 1999), its effects on germ cells are largely mediated by Sertoli cells.

3 CHARACTERISTIC FEATURES OF HAPLOID PHASE OF SPERMATOGENESIS

3.1 Haploid cell specific genes

Despite the fact that all the spermatids contain only a haploid genome; each spermatid will finally develop into fully maturing spermatozoa. When translation activity of spermatids are studied only few proteins are derived from the stored mRNAs transcribed early in the nucleus of spermatocytes, demonstrating that spermatids are transcriptionally and translationally very active cells (Hecht, 1990). This conclusion is further supported by the recent finding of many haploid cell specific gene products (Penttilä et al., 1995; Pereira et al., 1998). Moreover, the uneven sharing of sex chromosomes (X and Y) between spermatids has increased the general interest to study their function in spermatids. Hendriksen et al. (1995) have shown that certain Y chromosomal genes are highly transcribed in spermatids and control spermiogenesis (Delbridge and Graves, 1999). It has also been demonstrated that at least partial inactivation of X chromosome is essential in the normal spermiogenesis.

These findings suggest that at least the gene products of sex chromosomes and most likely haploid cell specific genes are actively transported between spermatids ensuring the synchronous development of the germ cells (Fawcett et al., 1959). Braun et al. (1989) showed with transgenic mouse strain that chimaeric gene product expressed only by postmeiotic cells, are evenly distributed between genotypically haploid spermatids. The functions of these haploid cell specific

genes are only elusively known, but most likely they are involved in the formation of haploid cell specific organelles such as sperm tail, chromatoid body and acrosomic system (Hecht, 1990).

3.2 Golgi complex and acrosomic system

In round spermatids one function of Golgi complex is in the development of acrosomic system, which contains hydrolytic enzymes involved in fertilization and penetration through zona pellucida (Hermo et al. 1980; Moreno et al., 2000b). In mammals, acrosome formation begins with the fusion of proacrosomal granules synthesized by Golgi complex, similar to the secretory granules in various other cell types (Susi et al., 1971). Because of the early biogenesis of the acrosome and its several hydrolytic enzymes, it is considered to be a lysosome-like organelle (Allison and Hartree, 1970). Acrosomic system formation can be subdivided chronologically into three main phases: Golgi, cap and acrosome phases. At early spermatids (stage I-IV) acrosomal vesicles attach to the nuclear envelope and the size of the acrosomic system grows as a result of the constant arrival and fusion of Golgi derived vesicles (Susi et al., 1970). Later the acrosomic system flattens and covers up to two-thirds of nucleus surface (stage V-VII). At stage VIII Golgi complex detaches from acrosomic system and remains suspended in elongated cytoplasm until it degenerates during step 16 spermatids (in rat).

Testis-specific proteolytic enzyme acrosin is present in sperm acrosome as zymogen form, proacrosin, which is activated to the mature enzyme acrosin during the so-called acrosome reaction. Previously it was believed that acrosin is needed in fertilization and penetration through zona pellucida (Bhattacharyya et al., 1979). However, male mice carrying the disruptive mutation (Acr$^{-/-}$) in the acrosin gene were fertile and the spermatozoa did not loose their ability to penetrate the zona pellucida and fertilize the egg (Yamagata et al., 1998). The same study suggested that the real function for acrosin is to accelerate the dispersal of acrosomal components during the acrosome reaction. Previous studies have also demonstrated that proacrosin gene transcription is first observed in pachytene spermatocytes (Kashiwabara et al., 1990; Kremling et al., 1991). However, there have been conflicting results concerning the time when the proacrosin mRNA is translated (Kashiwabara et al., 1990; Nayernia et al., 1994). Moreover, Flörke et. al (1983) have suggested that acrosin is routed into the acrosomal compartment at the very beginning of the acrosomic system formation. Although the Golgi complex has a crucial function in formation of the acrosomic system it is obvious that Golgi complex plays also a main role in the cytoplasmic granule trafficking in male germ cells (Lippincott-Schwartz et al., 2000; Moreno et al., 2000a).

3.3 Sperm tail formation

Mammalian sperm tail is a very complex structure involved in generating and regulating the flagellar beat. During the spermiogenesis, formation of sperm tail is one of the main processes, lasting from early spermatids until sperm release occurs. In the rat the first evidence of flagellum is seen in the step 2 spermatids. After migration of the centriole pair to the cell surface, one of the two centrioles form axoneme, a structure containing microtubules (9+2 arrangement) at the cell surface (Russell et al., 1990). The axoneme extends outward from the cell lumen and reaches the tubular lumen. Then the centriole pair moves from surface of the cell towards the nucleus where it inserts the nucleus surface at the opposite side to acrosomic system. Later accessory components, such as mitochondria, are added to the flagellum to form middle, principal and end pieces.

3.4 Chromatoid body

An evolutionarily conserved feature of germ cell differentiation is the large cytoplasmic organelles locating next to nucleus. During spermatogenesis this organelle is called chromatoid body. Moreover, totipotent somatic stem cells of planarians (so called neoplasts) contain chromatoid body in the cytoplasm supporting the conserved feature of this organelle (Hori, 1982). In Drosophila oocytes, an analogous organelle is called sponge body (Wilsch-Bräuninger et al., 1997) and in humans fetal oocytes yolk nucleus (Hertig & Adams, 1967). Recent investigations have suggested similar functions for these organelles of both sexes and planarian stem cells. Immunohistochemistry with antibodies against conserved germline-specific RNA-binding VASA proteins demonstrated that the yolk nucleus (Castrillon et al., 2000) and both the chromatoid bodies of mammalian spermatogenic cells (Toyooka et al., 2000) and totipotent neoplasts of planarians (Shibata et al., 1999) are VASA positive. However, the functions of these organelles are not clearly known.

Chromatoid body has been suggested to have a role in transport and storage of RNA in haploid cells. It has been demonstrated to contain radioactivity derived from tritiated uridine (Söderström and Parvinen, 1976a), actin (Walt and Armbruster, 1984), RNA (Figueroa and Burzio, 1998), histone H4 (Werner and Werner, 1995), germ cell-specific DNA and RNA binding protein p48/52 (Oko et al., 1996), hnRNPs and ribosomal proteins (Biggiogera et al., 1990) and mouse VASA-homologue (Toyooka et al., 2000). It moves rapidly in the cytoplasm of living early spermatids (Parvinen and Jokelainen, 1974) in both parallel and perpendicular fashion related to the nuclear envelope, suggesting for a transport function of haploid gene products (Parvinen and Parvinen, 1979). It is possible that the parallel movements over the haploid nucleus are needed for collection of gene products from various parts of the haploid nucleus from different chromosome territories (Cremer et al. 1993). The significance of the perpendicular component of the movement of chromatoid body has been more difficult to understand. Finally, it is obvious that these perinuclear organelles of both sexes have an important role during gametogenesis, since targeted disruption of VASA

or its homologue genes results in sterility and serious defects in germ cell development both during oogenesis and spermatogenesis (Styhler et al., 1998; Tanaka et al., 2000).

3.5 Cytoplasmic bridges

The diameters of the intercellular bridges increases during spermatogenesis (Weber and Russell, 1987). Spermatogonial bridges are 1 −1.3 μm, at step 1 spermatids the diameter of the bridge is approximately 1.8 μm and step 18 spermatids contain bridges with diameter of 3.0 μm. Molecular structure and the development of intercellular bridges in Drosophila germ cells are better understood than in mammals (Robinson and Cooley, 1996). Most widely studied model of cytoplasmic bridges (or ring canals) is ovary and nurse cells in Drosophila. Bohrmann and Biber (1994) have studied living cytoplasmic bridges during Drosophila oogenesis and demonstrated that only few (ca 30%) of the granules circulating in the immediate vicinity of the bridge actually were seen to pass through. This suggests that selection and regulation of material transport takes place in the cytoplasmic bridges. This may be due to the proteins found inside the cytoplasmic bridge. Interestingly, the mutation of a specific gene, cheerio (Robinson et al., 1997), prevents the expansion of cytoplasmic bridges during oogenesis, resulting obvious malfunction of cytoplasmic material sharing, decrease in the size of oocyte and female sterility, demonstrating the great importance of cytoplasmic bridges during gametogenesis.

4. THE LIVING CELL STUDIES

4.1 Background of the living cell study

In 1838 Matthias Schleiden, researcher of plant cells, and zoologist Theodor Schwann who studied nervous tissue of animals realized that all animals and plants are composed of cells and even eggs and sperm are cells. This was the beginning of cell theory, which was officially published in 1858, by a German pathologist, Rudolph Virchow. The starting point of studies of living cells was the invention of phase contrast phenomenon and microscope in 1934 by Fritz Zernike. The discovery of the phase contrast effect made it possible to study the motile organisms and the movements of cellular organelles. However, living cell studies did not proceed because of many remaining difficulties. To mention a few: 1) due to the lack of the effective photography equipments the observations achieved by studying the living cells remained quite subjective, 2) the phenomenon in the living cells are sensitive for the fixation procedures which makes it difficult to demonstrate the *in vivo* situations after fixation, 3) fixation procedures have developed considerably diminishing the need of living cell studies. However, recent technical advances in computers, digital imaging and biotechnology have increased the interest of scientists on living cell study. Concrete improvements have been the increased capacity and speed of the hardware of the computers, sensitivity of cameras and the discovery of green fluorescent protein (GFP). These

technical improvements have made it possible to store, analyze and share the real time observations of living cells. Finally, the recent development of internet has also increased the interest for living cell studies among many scientific journals.

4.2 Green fluorescent protein (GFP)

The jellyfish *Aequorea victoria* yields a natural green fluorescence which can be viewed in a dark environment. The green fluorescent protein (GFP) is the ultimate source of this green bioluminescence and is generated after calcium binding to photoprotein aequorin (Shimomura et al., 1962). Purified GFP protein contains 238 amino acids, absorbs blue light (395-470 nm) and emits green light (peak emission at 509 nm; Prasher et al., 1992). Chalfie et al. (1994) demonstrated that complementary DNA of GFP can be transferred into prokaryotic (Escherichia coli) and eukaryotic (Caenorhabditis elegans) cells to monitor gene expression in living cells. Subsequently, green fluorescent protein has been demonstrated to be an ideal transgene expression marker, which allows a noninvasive study and accurate localization of known gene products inside the living cells (Wang & Hazelrigg, 1994, Yeh et al., 1995).

Many different GFP molecules have been generated that are more useful in several respects than wild-type GFP. Both red-shifted (yellow) and blue GFP have been described in the literature (Tsien, 1998). Okabe et al. (1997) generated the first "green" transgenic mouse strain, where EGFP (enhanced form of GFP) was expressed under control of chicken beta-actin promoter. In this mouse strain most of the tissues, especially muscle and pancreas, expressed very intense fluorescence when studied under 488 nm ultraviolet excitation light. Histological analyses showed no abnormalities demonstrating that EGFP is a nontoxic marker *in vivo*. Because of the soluble nature of EGFP molecule this marker locates homogenously inside the cells.

4.3 GFP marker in the monitoring of protein localization, intracellular trafficking and spermatogonial stem cell transplantation

GFP techniques have added our knowledge about the kinetics and molecular basis of intracellular organelle traffics. Particularly studies about the roles of Golgi complex and interactions between different compartments of Golgi complex (cis- and trans face) and endoplasmic reticulum (ER) have obtained great benefits about new GFP techniques (Lippincott-Schwartz et al., 2000). In 1997 Presley et al. showed visually for the first time with viral glycoprotein (ts045 VSVG) tagged with GFP the ER-to-Golgi transport. Moreover, specific microtubule inhibitors (eg. nocodazole) and Golgi complex disrupting agents have increased the knowledge about the regulation of intracellular trafficking (Lippincott-Schwartz et al., 1989).

Nakanishi et al. (1999) have produced transgenic mouse line where an enhanced green fluorescent protein (EGFP) is expressed under control of proacrosin

promoter. Moreover, EGFP was fused with proacrosin signal peptide sequence, which leads EGFP markers into the normal subcellular localization of proacrosin inside the cells. Thus, the relative expression levels and transportation of proacrosin protein inside the living male germ cells are accessible for studies under fluorescence microscope. Because proacrosin is expressed only in the spermatogenic cells, there is a possibility to study the translation, transportation and localization of proacrosin in different developmental phases during spermatogenesis.

An effective *in vivo* assay system has recently been developed where mouse spermatogonial stem cells are transplanted into infertile mouse testis to achieve a normal spermatogenesis in the recipient testis (Brinster and Zimmermann, 1994). In kinetic analyses of transplanted spermatogonial stem cells, it has been demonstrated that qualitatively complete spermatogenesis was observed after 2-3 months (Nagano et al., 1999). Recently, mouse spermatogonia carrying both chicken β-actin-EGFP (pCXN-eGFP; Okabe et al., 1997) and acrosin-EGFP (Acr3-EGFP; Nakanishi et al., 1999) transgenes were transplanted into infertile recipient mouse testis (Ohta et al., 2000). The specific expression of these markers can be used for the identification of differentiation steps of male germ cells in transplanted testis *in vivo*.

AIMS OF THE PRESENT STUDY

In mammals there exist several tissues (such as skin, bone marrow, ovaries, testis) where continuous, highly organized cell differentiation is obligatory for the survival of the individual. This regeneration is maintained by the tissue specific stem cells. However, the lack of effective in vitro or in vivo systems in which the normal development of the cells can be monitored have severely complicated the studies of the regulation of tissue specific cell differentiation. Spermatogenesis provides an excellent model of cell differentiation, where different genes are expressed at certain phases in a strictly coordinated fashion. Therefore it provides possibilities for studies of stem cell niches. Recent discovery of testis specific genes, the development of GFP transgene technology and recently developed effective *in vivo* assay system for studying the differentiation of male germ cells in infertile testis has opened new opportunities to study the male germ cell differentiation at molecular level.

The specific aims of the study were:

I.   To develop a method to study in living spermatogenic cells the expression and hormonal regulation of beta-actin by using EGFP transgene mice as a model.

II. To study the kinetics and regulation of acrosomic system formation *ex vivo* and *in vitro* by studying transgenic acrosin-EGFP mice.

III. To adopt squash preparation technique to study subcellular localizations of novel haploid cell specific gene (Shippo 1).

IV. To adopt living cell techniques to study the cell composition and to analyze the kinetics of stage formation in the infertile testis after transplantation of double-EGFP transgenic spermatogenic stem cells.

V.   To gain visual evidence about the haploid cell specific gene product sharing between genotypically haploid spermatids and to study the involvement of intercellular bridges, Golgi complex and chromatoid body in the material sharing.

MATERIALS AND METHODS

5.1 Animals and tissue preparation (I-V)

Either adult EGFP-transgenic mice (I,II, IV, Okabe *et al.,* 1997, Nakanishi et al., 1999), wild type mice (III, C57BL/6) or rat (Sprague-Dawley, V) were used as donors of the testes. They were housed in a cage with a light:dark cycle of 14:10 h and received water and food *ad libitum.* CO_2 asphyxiation was used to extinguish the animals for the experiments. The testes were removed and after decapsulation, the seminiferous tubules were dissected free from the interstitial tissue in a Petri dish containing phosphate-buffered saline (PBS) solution. The transillumination pattern and the living cells in the squash preparations were recognized as described previously (Parvinen & Hecht, 1981; Parvinen and Vanha-Perttula, 1972) and tubules at defined stages of the cycle (Oakberg, 1956; Leblond and Clermont, 1952) were selected for further analysis. All animal experiments were approved by the Turku University Committee of Ethics and Animals Experimentation (permission no. 848/98).

5.2 Squash preparation technique (I-V)

Tubule segments of 0.5-1 mm in length from defined stages were identified by transillumination, microdissected under a stereomicroscope and transferred with a pipettor (15 µl of medium) to microscope slides. A coverslip (24 x 24 mm) was placed over the tubule segment carefully avoiding the formation of air bubbles. The excess fluid was removed by blotting which allowed the cells of the seminiferous epithelium to float out. This was monitored under 40 x phase contrast optics to adjust a slightly flattened monolayer. The edges of the coverslip were then carefully sealed with paraffin oil in order to achieve a complete immobilization of the cells. The cells were identified and examined under phase contrast optics with a 100 x oil immersion objective (I-V) and their fluorescence intensities (I, II, IV) were measured.

5.3 Transplantation of double-EGFP transgenic stem cells (IV)

To obliterate the spermatogenic cells, busulfan was injected intraperitoneally at a dose of 40 mg/kg. The busulfan-treated males were used as recipients 4 weeks after injection. Double transgenic mice [C57BL/6TgN(acro/act-EGFP)OsbN01] carrying both acrosin/EGFP (Acr3-EGFP; Nakanishi et al., 1999) and pCXN-EGFP (Okabe at al., 1994) transgenes were used as donors of spermatogonial stem (Ohta et al., 2000). The cell suspension for transplantation was prepared by using a two-step digestion procedure (Ohta et al., 2000). Briefly, the testes of 7-day-old mice were placed in Dulbecco's modified Eagle's medium (DMEM) buffered with 20 mM Hepes at pH 7.3 containing collagenase type I (1 mg/ml) and hyaluronidase (1 mg/ml). They were incubated for 15 minutes at 37 °C, with manual agitation at 5-minute intervals. The seminiferous tubules were washed twice in calcium-free phosphate-buffered saline (PBS), and then incubated in PBS

containing 0.25% trypsin for 15 minutes at 37 °C, with manual agitation at 5-minute intervals. After adding fetal bovine serum of a half volume of the cell suspension and several flushes by a pipette, the cell suspension was filtered through a nylon mesh, 30 μm pore size, to remove large clumps of cells. The filtrate was centrifuged at 600 xg at 16°C for 5 min, and the cell pellet was then resuspended in injection medium (138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.1 mM KH_2PO_4, 0.1 mM EDTA, 5.5 mM glucose, 5 mg/ml bovine serum albumin, 100 μg/ml DNase I, and 0.4 mg/ml trypan blue) (Brinster and Zimmermann, 1994) at a concentration of 10^6 or 10^8 cells/ml. The transplantation was performed via the efferent ductules as previously described (Okawa et al., 1997). Approximately 10 μl of the donor cell suspension was transplanted into the seminiferous tubules of busulfan-treated mice.

5.4 Tissue culture (I, II, V)

Staged seminiferous tubule segments (0.5 mm) were isolated by transillumination-assisted microdissection. Both ends of the selected seminiferous tubule were examined by squash preparations to ensure the exact stage of the cycle (Toppari et al., 1988). Selected tubules were incubated for 48 h at 34°C in 100 μl of Ham´s F12/Dulbecco´s MEM (1:1)(Life Technologies, Grand Island, NY) in humidified atmosphere containing 5% CO_2. The medium contained 0.1% BSA and 10 μg/ml of gentamycin. Follicle stimulating hormone (1.0 and 10 ng/ml of human recombinant FSH, Org 32489; 10.000 IU/mg, Organon, Oss, The Netherlands) was used for the hormonal stimulation. Nocodazole (1.0, 10 and 100 μg/ml) and cytochalasin D (1.0, 10 and 100 μg/ml) were dissolved with DMSO or 94% ethanol to inhibit microtubule and actin related events, respectively. Brefeldin A (1.0 and 5.0 μg/ml), dissolved with 94% ethanol, was used to disrupt the Golgi complex.

5.5 Immunocytochemistry (II, III, V)

After observation of living squash preparation, it was snap frozen in liquid nitrogen and fixed with +4C ethanol (97%; III, V) or with 3% paraformaldehyde (II). Then the coverslip was removed and the slides were stored in cold PBS. Fixed cells were permeabilized with 0,5% Triton X for 10 min followed by two washes with PBS and PBS/gelatin for 5 min. The cells were then incubated either with monoclonal antibody TRA54 1:50 (Pereira et al. 1998; V), polyclonal anti-Mvh antibody (mouse VASA-homologue, Toyooka et al. 2000; V) 1:1000, anti-HSF-2-antibody 1:150 (Alastalo et al. 1998, V) or monoclonal anti-α-tubulin antibody 1:150 (clone DM 1A, Sigma, St. Louis, Missouri, II) for 1-10 h. Control slides were incubated with appropriate non-immunized animal serums. After two washes with PBS and PBS/gelatin the slides were incubated for 1-10h with fluorescein conjugated anti-mouse, -rat or -rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania).

5.6 Electron microscopy (V)

Tubule segments (ca 2mm in length) from stages I - IV of the cycle were isolated by transillumination (Parvinen and Vanha-Perttula, 1972), and fixed in 5% glutaraldehyde in s-collidine-HCl buffer (0.16 M, pH 7.4) at 20C, postfixed with 1% osmium tetroxide in 1.5% potassium ferrocyanide and embedded in Epoxy resin (Glycidether 100, Merck, Germany). They were sectioned at 70 nm with a Reichert E ultramicrotome (Reichert Jung, Austria). Uranyl acetate and lead citrate were used for staining of the sections, before examination with a Jeol 100 SX (Jeol, Tokyo, Japan) electron microscope.

5.7 Immunoelectron microscopy (V)

Seminiferous tubules of stage I-IV were fixed with 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.08M sodium cacodylate buffer containing 0.05% calcium chloride, pH 7.3, for 4 hours. After dehydration in ascending series of ethanol, samples were embedded in LR-White at 50°C for 72 hours. Sections of approximately 100 nm were cut with Reichert Ultracut E microtome and placed on nickel grids and incubated in the presence of monoclonal rat anti-TRA54 antibodies (Pereira et al., 1998) or non-immunized normal rat serum (control slides) in 0.01M phosphate buffer, pH 7.4, 0.1% BSA (Sigma), 60 minutes, 1:10. This was followed by goat anti-rat IgG conjugated with 15 nm olloidal gold (1:30, Electron Microscopy Sciences, Fort Washington, Pa) and counterstaining with uranyl acetate and lead citrate. Finally, samples were examined with a Jeol 100 SX (Jeol, Tokyo, Japan) electron microscope at 60 kV.

5.8 Image acquisition and analysis (I – V)

A Kappa CF 8/1 FMC CCD black/white video camera (Gleichen, Germany) was attached to a Leica DMRB phase contrast microscope (Wetzlar, Germany). Image sequences were directly digitized (I-V) and stored into a hard disk for 300 s at a rate of 4-6 pictures/s (V) using a FAST image grabbing system (FAST Multimedia AG, D80339 Munich, Germany). The frames from original AVI-files were first converted to bitmap (bmp) format. A custom-made image analysis programs developed for Windows95 platform were used in the analyses of granule and organelle movement by recording the granule position coordinates in consecutive frames (V) or to quantify EGFP fluorescence intensity from Windows bitmap greyscale images (I, II). The distances were determined in pixels, and converted to metric scale (328 pixels = 10μm). The distances, movement paths and velocities of the granules were plotted using Microsoft Excel spreadsheet program (version 97, Microsoft Corporation, USA).

To monitor FSH stimulation of beta actin-EGFP expression, whole culture plate was inserted into Victor[2] 1420 Multilabel Counter (Wallac, Turku, Finland) to analyze the temporal level of fluorescence at the different time points (I).

Fluorescence intensity at the beginning of incubation was selected as a basal value 1, and all subsequent measurements were related to this value.

5.9 Special methods in communication III

Clone β390 from previously described haploid germ cell specific cDNA library (Tanaka et al., 1994) was used as a probe to screen an adult mouse testis cDNA library in order to isolate its complete sequence (*shippo 1*). Total RNA for the northern blot analyses was extracted from various organs of C57BL /6 mice using RNAzol B (Tel-Test Inc., Friendswood, TX). To investigate the developmental changes of Shippo 1 gene expression in the mouse testis, RT-PCR (the Thermoscript reverse transcription-polymerase chain reaction system provided by Gibco BRL, Rockville, MD) was performed using total RNA of the testis at the ages of 2–5, 8, 17, 23, 29, and 35 days. For the immunohistochemistry and western blot analyses purified Shippo 1 recombinant protein expressed in E. coli was used as an antigen to raise polyclonal antiserum in rabbits.

RESULTS AND DISCUSSION

6.1 Stage specific expression of beta-actin EGFP in living spermatogenic cells I

We exploited a "green" transgenic mouse strain (Okabe at al., 1997), where EGFP (enhanced form of GFP) was expressed under the control of chicken beta-actin promoter to study beta-actin expression in the seminiferous tubules in living condition by transillumination and phase contrast microscopic techniques (Parvinen & Hecht, 1981; Toppari et al., 1988). Squash preparations made from stages X and XII of the cycle showed the brightest β-actin-EGFP fluorescence in step 10 and 12 spermatids. Lower intensities were found in pachytene and the lowest in the leptotene primary spermatocytes. Moreover, type A-spermatogonia had a relatively high intensity. After step 10, the nuclear EGFP-fluorescence intensity declined rapidly starting from the tip of the step 11 and 12 elongating nucleus. However, the cytoplasmic fluorescence intensity stayed relatively constant during late phases of spermiogenesis. The autofluorescence intensity of wild type mice were very low compared with β-actin-EGFP fluorescence and it did not interfere the measurements.

The present results agree with those of Waters et al. (1985), who demonstrated with Northern analysis that the level of β-actin-mRNA was highest in isolated round spermatids as compared with the levels in pachytene spermatocytes or residual bodies. Similarly, Hecht et al. (1986) showed with radiolabelling that the relative amount of β-actin protein increases between meiosis and late spermiogenesis. It has also been documented with two-dimensional electrophoresis that β-actin expression is higher in round spermatids, elongating spermatids and residual bodies compared to pachytene spermatocytes (Hecht et al., 1984). These results suggested that the β-actin transcripts are expressed at high levels in meiotic cells, stored inside the germ cells and translated predominantly in postmeiotic haploid cells. In our study the intensity of EGFP, which is expressed under chicken beta-actin promoter, rose from pachytene spermatocytes up to step 12 spermatids. This is in accordance with the results of Hecht and coworkers (1984, 1986), and suggests that the mRNA of this transgene promoter is translated in the same fashion as the normal beta-actin protein. However, the high expression of chicken beta-actin controlled EGFP in type A-spermatogonia and a slight decrease of fluorescence at steps 13-16 spermatids have not been described earlier.

Previous studies on actin localization in late spermatids and in spermatozoa are conflicting. Halenda et al. (1987) used rhodamine-phalloidin to stain actin filaments and demonstrated that staining and the presence of actin filaments in the region of the developing acrosome in guinea pig spermatids diminished as development progressed. Flaherty et al. (1986) used monoclonal antiactin antibody and NBD-phallacidin to label actin in spermatozoa in the bull, boar,

rabbit, human, rat, mouse, golden hamster, and guinea pig. They demonstrated that actin was present in sperm in all these species.

Kistler et al. (1996) have shown in rats that transition proteins 1,2 and protamine 1, are expressed in stage specific manner. Transition proteins appear during the period when spermatids undergo nuclear elongation and condensation. In our study the β-actin-EGFP fluorescence was highest in the cytoplasm of elongating spermatids (steps 12 and 13), when the expression levels of transition proteins 1 and 2 are also highest. It is possible that β-actin has role in the elongation of the spermatid nucleus. However, an interesting finding in this study was the puzzling localization of EGFP in the nucleus of different germ cells. In recent studies, GFP-molecule is also demonstrated to be transported into nucleus in a transfected cell lines cells (Samejima and Earnshaw, 2000; Giannini et al., 2000). Activity of EGFP in the nucleus of elongated spermatids lowers after step 12 spermatids. Timing is the same when the expression of protamine 1 increases (Kistler et al., 1996), suggesting that the compaction of the chromatin relocates the EGFP molecules out of the nucleus.

EGFP is an effective transgene expression marker and it is nontoxic for animals (Okabe et al., 1997). Transgenic mouse strain used in this study was also normal, healthy and fertile. Also the morphology of all germ cells was normal despite the EGFP expression. This study shows for the first time, that it is possible to follow gene expression in a noninvasive way in living spermatogenic cells, accurately identified by phase contrast microscopy of live cell squashes.

6.2 Stage specific stimulation of FSH of the beta-actin EGFP I

Follicle stimulating hormone (1.0 ng/ml) stimulated β-actin-EGFP expression after 4 hours of incubation in stage I-VI of the cycle. After 24 hours both stage I-VI and stage VII-VIII were sensitive for FSH. No significant differences between FSH-stimulated and unstimulated tubules was found at any time point at stage IX-XII. After 48 h of incubation, the β-actin-EGFP intensity was declined, and the stimulatory effect of FSH at stages I-IV and VII-VIII was no longer present. Incubations with 10 ng/ml of FSH did not have significant effects. These results demonstrate that expression of beta-actin-EGFP in stages I-VI is more sensitive to FSH stimulation than other stages, suggesting for a regulatory effect of FSH on the cytosceleton. At stages VII-VIII FSH stimulation was also found, whereas stages IX-XII were insensitive to FSH. This is in accordance with the concept of stage-specific action of FSH during the cycle of the rat seminiferous epithelium (Kangasniemi et al., 1990, Parvinen, 1993), and first observation in the mouse to support an existence of similar stage-specific FSH-regulation.

This study shows that transillumination-phase contrast microscopic methods applied to EGFP transgenic animals give new information about the temporal regulation and expression of gene products in living cells at an accuracy level not easily reached by other techniques.

6.3 Acrosin expression and accumulation into Golgi complex II

Nakanishi et al. (1999) have produced transgenic mouse line where an enhanced green fluorescent protein (EGFP) containing proacrosin signal peptide is expressed under control of proacrosin promoter (Acr3-EGFP). We have adopted this mouse model to study the relative expression levels and transportation of proacrosin protein inside the living male germ cells. Our study shows that proacrosin promoter is first expressed in pachytene phase of first meiotic division at stage IV of the cycle of the seminiferous epithelium, and in all steps of meiosis and spermiogenesis beyond that point. The accumulation of proacrosin into the Golgi complex is first seen in diakinetic primary spermatocytes and finally it concentrates into proacrosomic granules during step 2 of spermiogenesis. A cloud-like accumulation of EGFP at the cis-edge of the Golgi complex is seen in step 3 spermatids with confocal microscope (Fig. 3). Proacrosin is finally accumulated into developing acrosomic system. Our study support the suggestion of Flörke et al. (Florke et al., 1983) that acrosin is routed into the acrosomal compartment at the very beginning of the acrosomic system formation.
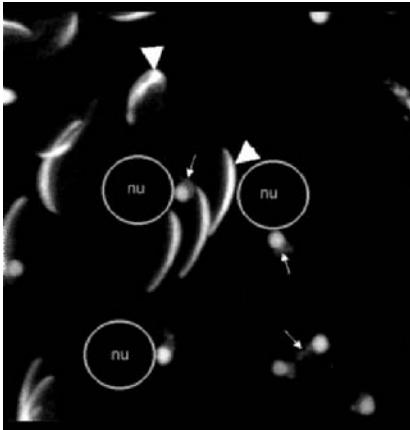

Figure 3. Confocal image from the living stage III spermatids (Figure 3.avi). A cloud-like accumulation of EGFP at the cis-edge of the Golgi complex (arrows) is seen next to nucleus (nu). Acrosomic system of elongated spermatids are pointed with arrowheads.

When seminiferous tubules of stage I are incubated for 48h in control culture conditions, step 1 spermatids differentiated to step 3. In the presence of 1.0 µg/ml of brefeldin A (Golgi complex disrupting agent), the cytoplasm of spermatids was EGFP positive but accumulation of EGFP into acrosomic system did not occur. 5.0 µg/ml of brefeldin A was toxic. This experiment demonstrates the importance of Golgi complex in acrosomic system development. Although the cytoskeleton remains intact the intracellular transportation of acrosin in spermatids is inhibited after brefeldin A treatment.

6.4 The roles of cytoskeleton and FSH in acrosomic system formation II

Previously, little information has been available about the dependence of the cytoskeletal components on development of the acrosomic system. In the present study we demonstrate that 10 µg/ml of nocodazole prevented the incorporation of acrosin and formation of acrosomic system in most spermatids. However, some spermatids showed an EGFP positive acrosome like structure that was enlarged in size and disconnected from the nucleus, suggesting that attaching of acrosomic system to the nucleus is dependent on microtubules. Incubation of stage I cells for

48 h with 10 µg/ml of cytochalasin D did not affect EGFP-proacrosin signal peptide transportation inside the spermatids. No effect was found when 1.0 µg/ml of nocodazole or cytochalasin D was used, and 100 µg/ml of both drugs induced a general toxicity for cultured germ cells. This data demonstrate that particularly the early formation of the proacrosomic granules is dependent on microtubules, whereas the formed acrosomic system seems to be less sensitive; acrosomic systems of step 3 and 15-16 spermatids tolerated the incubations with nocodazole. Therefore, acrosin transportation in male germ cells from ER to Golgi is microtubule dependent and supports the previous concept that was based on cell culture studies (Presley et al., 1997).

After 48 h of incubation with 10 ng/ml of FSH, EGFP accumulated normally into the acrosomic system but the size of the organelle remained smaller. Under phase contrast optics acrosomic system appeared very dense and dark when compared with control cells. With 1.0 ng/ml of FSH these effects were not obvious. α-tubulin immunohistochemistry demonstrated that in the cells incubated in control medium microtubules located mainly peripherally, next to the plasma membrane. In contrast, FSH enhanced the formation of microtubules in spermatids. Staining of background level was achieved by incubation with non-immune serum. Previous experiments utilizing transillumination technique have revealed changes among several parameters related to the action of FSH and androgens during the cycle of the seminiferous epithelium (Parvinen, 1979). This indicates that the function of the Sertoli cells is influenced by the associated spermatogenic cells. FSH has a preferential action during stages I-V of the cycle. Although the precise functions of FSH in cellular level have remained elusive (Simoni et al., 1999), its effects on germ cells are largely mediated by Sertoli cells. Our study supports the concept that FSH has either indirect or direct action on the microtubules and acrosomic system formation in early spermatids.

Briefly, the present study demonstrates that microtubules are vital for acrosin transportation and acrosomic system formation and location. FSH increased the density of the acrosomic system, which was most probably due to increased formation of microtubules. Furthermore, transgenic mouse strain where EGFP is expressed under desired promoter is a potentially important tool to study functions, regulation, localization and transportation of proteins during differentiation of male germ cells.

6.5 Subcellular localization of haploid gene product Shippo 1 protein during spermiogenesis (III)

To investigate the appearance of novel haploid cell specific gene, SHIPPO 1, in individual germ cells, the squash preparation method was adopted to study stage specific localization of the studied protein in developmentally different male germ cells after immunohistochemical staining with anti-SHIPPO 1 antibody (Fig. 4). Due to its presence in the sperm tail, the new protein was named as SHIPPO 1, from the Japanese word for ''tail''. SHIPPO 1 was absent in premeiotic germ cells

(spermatocytes) (Fig. 4, A and a). First detection was found in Golgi-complexes of early haploid cells (round spermatids at step 4; Fig. 4, B and b). In late round spermatids, where the tail precursor is visible and the cells have acquired a polarized asymmetry, the signal was detected clearly in the Golgi region, which by then had drifted apart from the acrosome vesicle and also in some of the vesicles scattered in the cytoplasm (Fig. 4, C and c). It is interesting that a limited region of the membrane in these cells was also marked, especially the posterior area, where the new tail is erupting. As the elongating spermatid approaches the top layer of the tubular epithelium, the cytoplasm shrinks and the development of the major structures of the spermatozoa is almost complete. At this terminal stage of spermatid maturation, SHIPPO 1 protein was restricted to the tail (Fig. 4, D and d). In the mature sperm in the epididymis, positive signal was also confined to the tail region (Fig. 4, E and e).
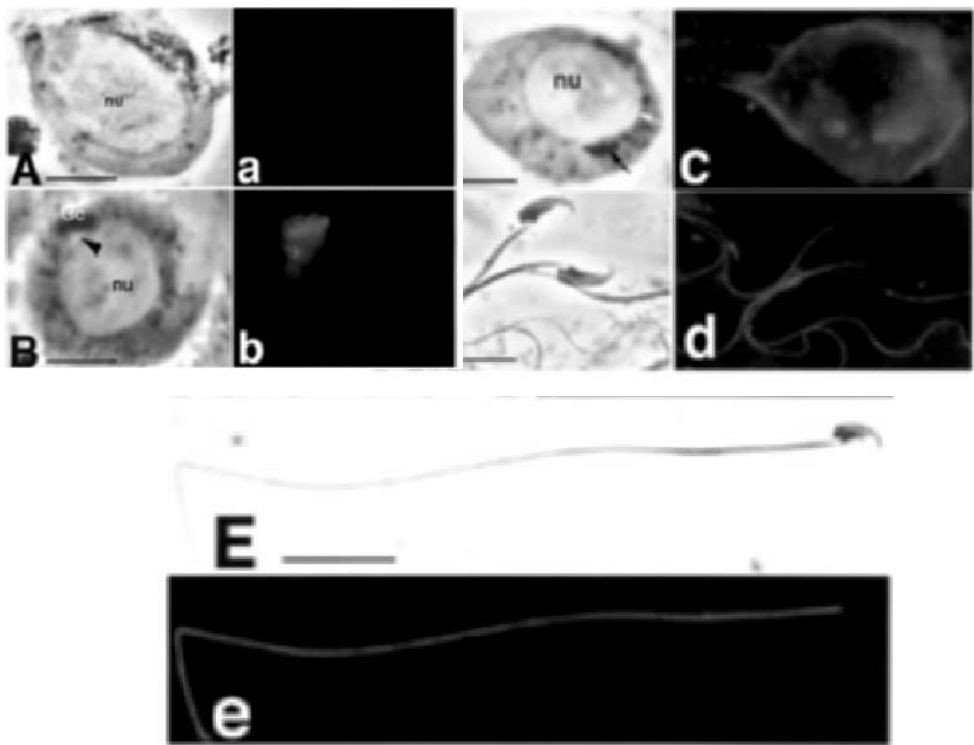

Figure 4. Immunostaining of individual germ cells isolated from mouse seminiferous tubules at specific stages of spermatogenesis (**A**, **a**, **B**, **b**, **C**, **c**, **D**, **d**) and caudal epididymal sperm (**E**, **e**). **A**, **a**) Pachytene spermatocyte isolated from seminiferous tubules at stage VIII. **B**, **b**) Early round spermatid at step 4–5 (arrowhead, acrosome vesicle). **C**, **c**) Round spermatid at step 8 (black arrow, Golgi complex, white arrow, acrosome vesicle, arrowhead, tail). **D**, **d**) Elongated spermatids or spermatozoa at step 16. **E**, **e**) Mature spermatozoa in the caudal epididymis. Capital letters, phase contrast microscopic images; small letters, fluorescent microscopic images obtained using rhodamine-labeled secondary anti-rabbit IgG; nu, nucleus; Gc, Golgi complex.

Apart from the axoneme and its associated proteins, the flagellum consists of 2 exclusive cytoskeletal components; the fibrous sheath (FS) in the principal piece,

and the outer dense fibers (ODFs) in the middle and principal pieces of the sperm tail. The cloning and characterization of ODF and FS components, once believed to be basically formed by actin and intermediate filament proteins (Kierszenbaum et al., 1996; Eddy et al., 1991), have often revealed a uniqueness and lack of homology with previously described cytoskeletal proteins (Carrera et al., 1994; Shao et al., 1999). The finding that these structures are targets of kinases and the involvement of Ca++ ions and cAMP has triggered a reconsideration of the classical cytoskeletal function in terms of a more active role in signaling and influencing flagellar motility (Carrera et al., 1994; Si and Okuno, 1993). In the present study a new haploid-specific sperm tail protein, SHIPPO 1, was cloned and characterized from cDNA library. The 93% identity in predicted amino acid sequence between mouse and human SHIPPO 1 suggests that these conserved proteins are indispensable for the successful male germ cell differentiation. In the present study a method where living spermatogenic cells are accurately recognized in squash preparations proved very efficient in studying the subcellular localizations of male germ cell specific proteins after immunohistochemical analyses. Moreover, the starting point during spermiogenesis, the Golgi complex localization, transportation and later localization in the tails of spermatids could be demonstrated with this method.

6.6 Analyses of the seminiferous epithelial cycle of double EGFP transgenic mouse testis (IV)

Recently, double EGFP transgenic mouse strain carrying both chicken β-actin-EGFP (pCXN-EGFP; Okabe et al., 1997) and acrosin-EGFP (Acr3-EGFP; Nakanishi et al., 1999) transgenes were generated (Ohta et al., 2000). We performed qualitative and quantitative analyses of differentiating male germ cells in the double EGFP transgenic mice. Different stages of seminiferous tubules of the adult double EGFP transgenic mice were distinguished by the transillumination technique (Parvinen and Hecht, 1981). From squash preparations differentiation of germ cells was identified according to the morphological criteria (Oakberg, 1952; Parvinen and Hecht, 1981). Specific patterns of EGFP fluorescence were used as additional aids for classifying the differentiated germ cells. pCXN-EGFP fluorescence varies in the developmentally different male germ cells and is very active in spermatogonia and spermatocytes. Acrosin-EGFP labels the developing acrosomic system and helps to identify the steps of spermiogenesis. Because of these differences in the timing of EGFP activity during spermatogenesis all germ cells were labeled with EGFP i.e., spermatogonia or spermatocytes were labeled with chicken β-actin EGFP, whereas spermatids were detected with Acr-EGFP transgene marker which labels the acrosomic system during spermiogenesis. Application of these useful markers facilitates us to study the detailed analysis of the stage of the seminiferous epithelial cycle together with the identification of the differentiated germ cells. Quantitatively and qualitatively spermatogenesis was normal in every stage of the cycle in the double EGFP transgenic mice.

6.7 Colonization of infertile mouse seminiferous tubules after transplantation of double EGFP transgenic spermatogonial stem cells (IV)

A technique where spermatogonial stem cells are successfully transplanted into infertile mouse testis has previously been described (Brinster and Zimmermann, 1994). By using double EGFP labeled transgenic mice (Ohta et al., 2000) as donors we have studied stem cell settlement, differentiation and the kinetics of colonization *in vivo*. Moreover, the establishment of the stage of differentiation in newly developed colonies at different time points was observed. Two months after transplantation colonized cells in the seminiferous tubules were identified by their fluorescence. The average length of the colony was 1.3 mm. The most advanced germ cells were haploid spermatids found typically in the middle of the EGFP positive colonies, although only spermatogonia together with occasional spermatocytes were found at the ends of the colonies. When more than two different parts of a colony were studied, cell association was usually the same in one colony and was similar to that of a normal stage. However, the stage of the cycle was not the same in different colonies. In some colonies, majority of the most advanced type of spermatids were at steps 6-7 but no elongated spermatids existed indicating that the germ cell differentiation was still not accomplished by two months after transplantation. Furthermore, a slight deviation from the cell association of normal seminiferous epithelial stages was also observed in some colonies i.e., some spermatids in more advanced step of differentiation seemed to appear later than normal. These results indicate that the cell association in one colony is similar, but independent of other colonies even in the same seminiferous tubules. Moreover, the accomplishment of different stages of the cycle requires more than 2 months after transplantation.

The average length of the colonies was 3.4 mm after 3 months after transplantation. The cell associations in every part of a colony were similar to that of the normal stage. The average increase in the length of transplanted colonies was estimated to be around 1.1 mm per month from 3.4 mm in 3 months after transplantation. Although this estimation is compatible with previous results (Ohta et al., 2000) increase in colony length by 2 months was approximately a half of this value (0.6 mm). Furthermore, many colonies were more than 2-3 times longer than the shorter ones. The average colony length should be approximately 3 mm or a little shorter in 3 months after transplantation if the colonies remain separated. Actually, the length of some of the observed colonies was more than 3.4 mm suggesting that some colonies were derived from the fusion of neighboring colonies, which then developed synchronously. Even after elongation of the colony to more than 4 mm in 3 months, only one type of cell association was found, although the length or the size of one stage segment of normal seminiferous tubules was estimated to be 0.4 to 3.2 mm in rats (Ismail et al., 1990). These findings indicate that the development inside the colony is synchronized. Furthermore, spermatogenesis seemed to be completed in 3 months after transplantation since we could observe a substantial number of elongated spermatids in all colonies. The interaction between newly settled spermatogonia

and the recipient Sertoli cells may first induce a proliferation of spermatogonia and then promote their differentiation (Ohta et al., 2000). This is supported by the observation that differentiation mainly occurs in the middle portion of the colony, while spermatogonia proliferate in the periphery. It is obvious that a certain number of spermatogonia is needed to form colonies large enough to allow differentiation.

6.8 Synchronization of germ cell differentiation and stage forming process in transplanted colonies (IV)

Three months after injection of high concentration (10^8 cells/ml) of testicular germ cells, we could observe many long (12.4 mm in average) fused colonies. Even in these fused colonies we observed a single cell association, indicating synchronization development of fused colonies. Almost all segments in one colony showed very similar associations of differentiated germ cells, although some colonies deviated a little from the normal pattern. These results indicate that the stage of the seminiferous tubules in the whole length of the large colony is synchronized although some of them begin to loose before disclosing physiological differentiation stages of seminiferous tubules. Six months after transplantation, however, the segmental dissection of the colonies revealed more prominent development into different cell associations. These results indicate that synchronization of the stage differentiation would take place at the time of colony fusion and then the deviation from the synchrony starts causing the wave of the seminiferous epithelium.

It is obvious that some regulatory mechanism(s) exist to maintain the synchronized development of male germ cells in the transplanted colonies. In our study, more than half of the colonies showed the cell association of stage VI-IX. As this phase is the point of nuclear elongation of round spermatids, it might be sensitive for the local factors secreted by Sertoli cells. This may cause some retardation at this stage and may act as a synchronizer in transplanted colonies. This notion is supported by *in vitro* observation that elongation of step 8 spermatids was impossible in culture conditions in rat (Toppari and Parvinen 1985). This might also explain the low amount of elongated spermatids in transplanted mouse testis even one year after transplantation (Russell et al., 1996). As Sertoli cells are known to regulate the proliferation and differentiation of germ cells, synchronized development of transplanted colonies may also be controlled by the Sertoli cells. This hypothesis is still supported by the recent finding that Sertoli cell function is cyclically controlled by maturing male germ cells (Parvinen, 1993) and also by differentiated spermatogonia (Russell et al., 1996). GDNF (Meng et al., 2000) and some other factors secreted by the Sertoli cells may regulate the proliferation and differentiation of spermatogonia to potentially synchronize germ cell differentiation in the fused colonies. It takes at least 6 months to develop the normal cyclic function of the Sertoli cells, and the fusion of adjacent colonies may be an important prerequisite for the normal cycle of the seminiferous epithelium to develop.

In the present study (IV) we have developed a new method to study the differentiation of male germ cells in a normal and transplanted testes where all male germ cells can be identified more accurately by morphological criteria of phase contrast microscopic image and in combination with EGFP fluorescence in live condition. We have also demonstrated that each colony in transplanted seminiferous tubules developed independently and maintained a typical stage-dependent cell associations for a very long time after transplantation. Furthermore, if more than 2 colonies are fused, each stage of the cycle is adjusted to one stage and synchronized. Some of the recently cloned testis specific genes or some stage specific paracrine factors may control this phenomenon. Double-labeled EGFP transgenic spermatogonia combined with accurate identification of living male germ cells is more sensitive to identify the differentiation of spermatogonial stem cells than the usual transillumination technique and is a good tool to study the mechanism of testicular germ cell differentiation and development of the stages of the cycle in the seminiferous epithelium.

6.9 Round spermatids share haploid cell specific gene product (TRA54) via cytoplasmic bridges (V)

Stable cytoplasmic bridges (or ring canals) connecting the clone of spermatids are assumed to facilitate the sharing of haploid gene products and synchronous development of the cells. We have visualized these cytoplasmic bridges under phase contrast optics and recorded the sharing of cytoplasmic material between the spermatids by a digital time-lapse imaging system *ex vivo*. The cytoplasm of living early spermatids contained a multitude of granules with diameters of approximately 0.5 μm. They moved along defined paths in a non-random fashion in varying speeds and had frequent contacts with each other and with larger organelles, such as Golgi complex and the chromatoid body. Some of these granules were moving in the vicinity of the bridges, but only 28% of those entering the bridge were actually transported into the neighboring cells. The average speed of the granules decreased significantly during the passage. We focused our interest in granules that were close to the intercellular bridges.

The diameter of the bridges was 1.9 – 3.0 μm. A characteristic step 2 spermatid, where a small granule migrates through the bridge and moves back and forth through the cytoplasmic bridge during 62.05 s observation time is seen in figure 5. The slowing down of the movement at the canal entrance and in the vicinity of the chromatoid bodies is evident. The average velocity of the granules transported through the cytoplasmic bridge was 0.44 μm/s inside the cytoplasm and the average transit velocity inside the cytoplasmic bridge was 0.23 μm/s, which is approximately 47.7% lower than that in the cytoplasm, $p < 0.001$.
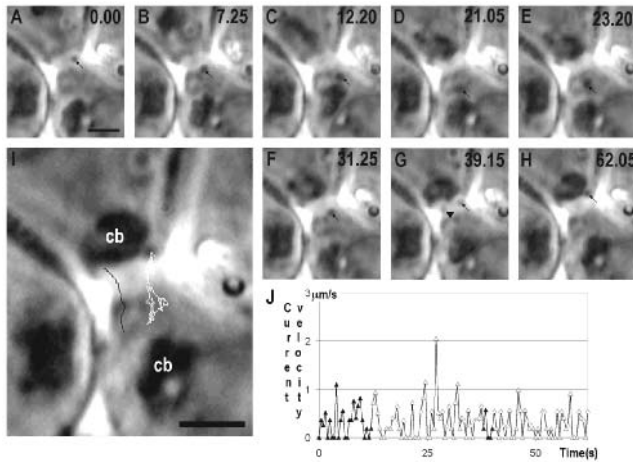
Figure 5. A successive series of time-scaled (in seconds, upper right corner) of images (A-H) of step 2 spermatid ex vivo, showing a two-directional movement of granules through the cytoplasmic bridge (Figure5.mov). Representative positions of the granule are shown by arrows. The path of movement is shown by white line at panel I. Another granule (arrowhead in G), traced by black line in I shows how it dissociate from the vicinity of chromatoid body and moves unidirectionally over the bridge. The changing velocity of granule at various time points is summarized in panel J; the movement inside the bridge is indicated with solid squares. cb = chromatoid body

To study the content of transported granules, germ cell-specific markers and antibodies against cytoskeleton and haploid cell specific gene products were used. Haploid cell specific monoclonal antibody TRA54 was localized in the granules migrating through the cytoplasmic bridge. Translation of antigenic epitope (sugar moiety) of TRA54 starts at early spermiogenesis. Golgi complexes of step 1 – 3 spermatids were highly TRA54 positive. Accumulation of TRA54 into acrosomic system is clearly seen in step 3-5 spermatids. Medulla and trans element of Golgi complex of early round spermatids were TRA54 positive in immunoelectron microscopic analysis. To study whether the transported granules seen *ex vivo* contain TRA54, the same cell was studied after fixation and double immunostaining with TRA54 and Mvh (Mouse VASA-homologue, Toyooka et al., 2000). The same granule that was seen to move through the cytoplasmic bridge between living cells, was TRA54 positive after immunostaining (Fig. 6). The exact localization of TRA54 positive granule inside the cytoplasmic bridge was confirmed by immunoelectron microscopy and confocal laser scanning microscope. In the elongated spermatids TRA54 was localized at the edge of the acrosomic system. This is the first direct demonstration of a haploid cell specific gene product transported through cytoplasmic bridges in mammalian spermatogenesis.
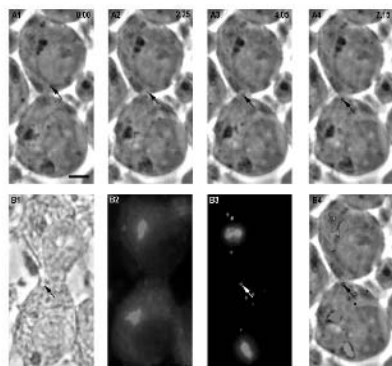

Figure 6. A successive time-scaled series of phase contrast images ex vivo showing granule (arrows) transport between neighbor spermatids (A1-4, Figure 6.avi). The time points in seconds are indicated at upper right corner. B1: phase contrast image from the same cells as in A after quick fixation, immunoreaction of anti-Mvh (mouse VASA-homologue; B2), TRA54 (B3), and superimposed image (B4) where granules and Golgi complex were TRA54 positive (green) and chromatoid body anti-Mvh positive (red). The same granule passing through the cytoplamsic bridge ex vivo (A) was TRA54 positive (arrows in B3 and B4).

6.10   The function of chromatoid body (V)

Male germ cell specific subcellular organelle, chromatoid body, is believed to have a role in storage and transport of haploid cell specific gene products (Söderström and Parvinen 1976a). We have demonstrated that this organelle moves back and forth through the cytoplasmic bridge between neighboring spermatids with transient contacts with nuclei of both spermatids (Fig. 7). Location of chromatoid body inside the cytoplasm and inside the cytoplasmic bridge was also studied after double immunostaining with Mvh (Mouse VASA-homologue) and TRA54 antibodies. The locations of the chromatoid body next to the nucleus, inside the cytoplasmic bridge and next to the nucleus of neighbor spermatid were demonstrated. The transient position of the chromatoid body inside the cytoplasmic bridge was confirmed also by electron microscopy. Moreover, with the snap frozen electron microscopy we were able to show a very close relationship between nuclear pore complex with a large contact area between the chromatoid body and the nuclear envelope.
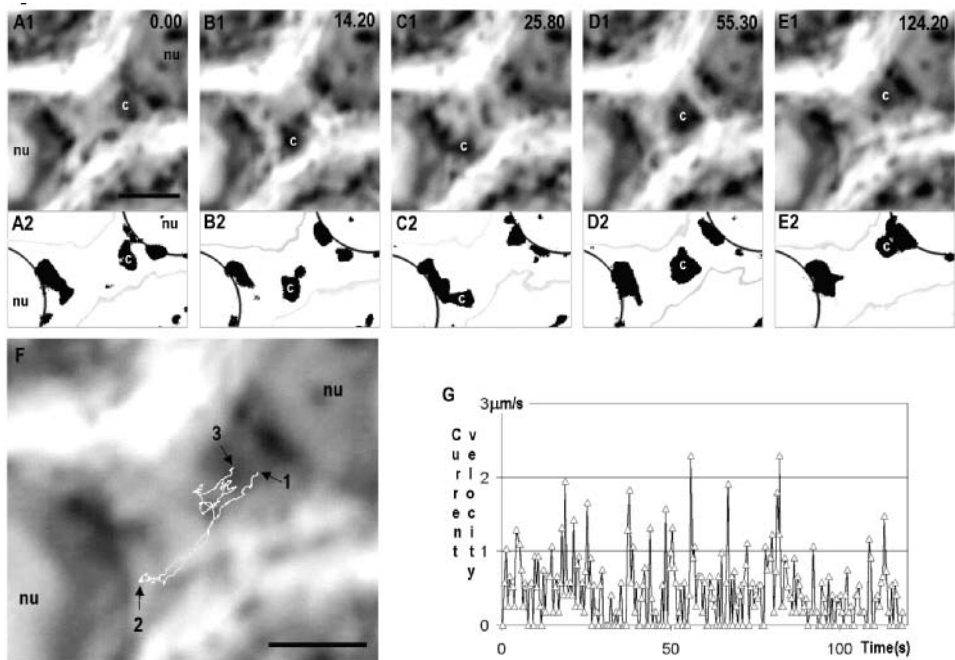

Figure 7. Time-scaled (upper right corner in seconds) series of phase contrast images showing two-directional movement of the chromatoid body (c) between two step 1 spermatids (A1-E1) through the cytoplasmic bridge (Figure7.mov). The same series is thresholded in A2-E2 to show in detail the chromatoid body and associated granules at the nuclear (nu) envelope. Moreover, in threshold image series the edges of the cells (gray) and nucleus (black) are presented. The movement path of the centroid of the chromatoid body is shown in panel F. Arrow 1 shows the starting point of the movement and arrow 2 the point where chromatoid body was close to the nucleus of the lower cell. Arrow 3 indicates the end point of chromatoid body movement during the 124.2 s recording period. The velocity of chromatoid body at various time points is shown in panel G. Bars = 2µm.

Previous time-lapse cinemicrographic observations have shown two main components of the rapid movement of the chromatoid body in early spermatids,

directed either parallel or perpendicular in relation to the nuclear envelope (Parvinen and Parvinen, 1979; Parvinen et al., 1997). The significance of these movements is not clear but it is possible that the parallel movements over the haploid nucleus are needed for collection of gene products from various parts of the haploid nucleus from different chromosome territories (Cremer et al. 1993). The significance of the perpendicular component of the movement of chromatoid body is more difficult to understand. However, the demonstration that chromatoid body moves through the intercellular bridge suggests this type of movement to be significant. Finally, our electron microscopic study demonstrated, for the first time, the location of the chromatoid body inside the cytoplasmic bridge between two spermatids. Previously, the special RNA rich structure called sponge body has been found from Drosophila oocytes (Wilsch-Bräuninger et al., 1997). It is located inside the nurse cells, inside the oocytes and inside the cytoplasmic bridge between nurse cells and oocytes, suggesting that this subcellular structure is transported between these cells. Therefore, the authors proposed that sponge body is needed in assembly and transport of mRNAs during Drosophila oogenesis, hinting at a number of similarities between chromatoid body and sponge body.

6.11 The roles of Golgi complex and microtubules in gene product sharing between spermatids (V)

To study the role of the Golgi complex in TRA54 translation, 48 hours *in vitro* incubation study was performed. Tubule segments (0,5 – 1 mm) from stage I of the cycle were selected for incubation. Under the control culture conditions, step 1 spermatids differentiated to step 3 during 48h; acrosomic system and TRA54 positive granules inside the cytoplasm were seen. However, if stage I seminiferous tubules were incubated for 48h in the presence of 1.0 µg/ml of brefeldin A, the spermatids showed no labeling with TRA54, but the chromatoid body remained intact and showed normal Mvh immunoreaction. Also no TRA54 positive granules were visible inside the cytoplasm after disrupting the Golgi complex. This finding emphasizes the role of the Golgi complex in male germ cell differentiation and in the transport of gene products between the spermatids. The lack of TRA54 immunostaining inside the cytoplasm after addition of brefeldin A is somewhat surprising, since the level of TRA54 labeling should increase inside the cytoplasm of the spermatids as brefeldin A inhibits the protein transport from the ER to the Golgi complex (Lippincott-Schwartz et al., 1989). Under immunoelectron microscope the TRA54 labeling is present only at the trans face of the Golgi complex. It is known that cisternae of Golgi complex are highly organized as a series of processing compartments: the phosphorylation of oligosaccharides takes place in cis-face and completion of glycosylation in trans-face. Also the sorting of proteins according to their final destination is performed at the trans-face of the Golgi complex. Based on these data it seems that TRA54 is processed in trans face of the Golgi complex of early spermatids either by glycosylation or alteration of configuration to recognized glycoprotein. These findings demonstrate that the Golgi complex has a novel role during spermiogenesis by sorting the granules needed not only for formation of

acrosomic system in one spermatid but possibly in several ones.

To study the roles of microfilaments and microtubules in cytoplasmic organelle movements, seminiferous tubule segments of stage I of the cycle were incubated for 48 hours with microfilament inhibitor, cytochalasin D, and microtubule inhibitors, nocodazole and vincristine. Nocodazole and vincristine at concentrations of 10 μg/ml turned the non-random cytoplasmic granules movement into random Brownian motion (Fig. 8), and the chromatoid body disintegrated to form several small spheres. Electron microscopic analysis revealed that these spheres contained ribosome-like granules covered by a thin layer of chromatoid material. After incubation of stage I tubule segments for 48h with nocodazole (10 μg/ml), two types of spermatids developed: one showed TRA54 immunostaining in the Golgi complex while the other lacked TRA54 labeling. This suggests that microtubule inhibitors block the transportation of TRA54 between the spermatids. Ca 90% of the spermatids were TRA54 positive and 10% negative.
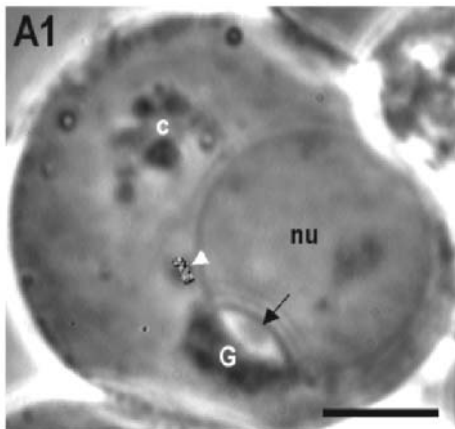

Figure 8. A1 shows step 3 spermatid after stage I seminiferous tubules are incubated for 48h in 10 μg/ml of vincristine. The chromatoid body (c) was disintegrated into several separate granules. Normal cytoplasmic granule movement was changed into random Brownian motion. Movement path of one granule is indicated at the white arrowhead (Figure8.mov). The morphology of the Golgi complex (G) remained intact under phase contrast optics. Acrosomic system is indicated with black arrow.

These results suggest that microtubules are involved in the normal integrity of chromatoid body, in its movements, and in the normal Golgi complex derived granule traffic in the cytoplasm of spermatids and between neighbor cells. Recently, Morales et al. (1998) presented immunocytochemical evidence that testis-brain RNA-binding protein (TB-RBP) moves from the nucleus to the cytoplasm and through intercellular bridges in rat spermatids, supporting the hypothesis that small granules may be involved in transport of gene products at mRNA level. TB-RBP has also been demonstrated to suppress in vitro the translation of mRNA and to bind specific mRNAs to microtubules (Han et al., 1995), which further supports the importance of microtubules in inter- and intracellular material transport.

CONCLUSIONS

I. Stages X and XII of the cycle showed the brightest β-actin-EGFP fluorescence in step 10 and 12 spermatids. Lower intensities were found in pachytene and the lowest in the leptotene primary spermatocytes. Moreover, type A-spermatogonia had a relatively high intensity. After step 10, the nuclear EGFP-fluorescence intensity declined rapidly starting from the tip of the step 11 and 12 elongating nucleus. β-actin-EGFP expression was most sensitive for follicle stimulating hormone (1.0 ng/ml) stimulation at stage I-VI.

II. Proacrosin promoter is first expressed in pachytene phase of first meiotic division at stage IV of the cycle of the seminiferous epithelium, and in all steps of meiosis and spermiogenesis beyond that point. The accumulation of proacrosin into the Golgi complex is first seen in diakinetic primary spermatocytes and finally it concentrates into proacrosomic granules during step 2 of spermiogenesis. After 48h incubation of stage I in control culture conditions, step 1 spermatids differentiated to step 3. In the presence of 1.0 μg/ml of brefeldin A (Golgi disrupting agent), the cytoplasm of spermatids was EGFP positive but accumulation of EGFP into acrosomic system did not occur. Nocodazole at 10 μg/ml concentration prevented the incorporation of acrosin and formation of acrosomic system in most spermatids, demonstrating that acrosin transportation in male germ cells from ER to Golgi is microtubule dependent and supports the previous concept that was based on cell culture studies (Presley et al., 1997). After 48 h of incubation with 10 ng/ml of FSH, Acr-EGFP accumulated normally into the acrosomic system but the size of the organelle remained smaller.
α-tubulin immunohistochemistry demonstrated that FSH enhanced the formation of microtubules in spermatids, supporting the concept that FSH has either indirect or direct action on the microtubules and acrosomic system formation in early spermatids.

III. Immunohistochemical staining revealed that novel haploid cell specific gene product, SHIPPO 1, was absent in premeiotic germ cells (spermatocytes). First detection was found in Golgi-complexes of early haploid cells (round spermatids at step 4). In late round spermatids, where the tail precursor is visible and the cells have acquired a polarized asymmetry, the signal was detected clearly in the Golgi region, which by then had drifted apart from the acrosome vesicle and also in some of the vesicles scattered in the cytoplasm. In the mature sperm in the epididymis, positive signal was also confined to the tail region.

IV. Qualitative and quantitative analyses of differentiating male germ cells in the double EGFP transgenic mice (Ohta et al., 2000) revealed that spermatogenesis was normal in every stage of the cycle in the double EGFP transgenic mice. When this mouse strain was adopted for the recipient of spermatogenic stem cells for the transplantation study stem cell settlement and differentiation could be observed in vivo under fluorescent excitation light. The average length of the colony was 1.3 after two months of transplantation and 3.4 mm 3 months after transplantation of

double EGFP labeled transgenic spermatogonia. Each colony transplanted into seminiferous tubules developed independently and maintained a typical stage-dependent cell associations for a very long time after transplantation. Furthermore, if more than 2 colonies are fused, each stage of the cycle is adjusted to one stage and synchronized. Finally, it takes at least 6 months to develop the normal cyclic function of the Sertoli cells, and the fusion of adjacent colonies may be an important prerequisite for the normal cycle of the seminiferous epithelium to develop.

V. We have visualized cytoplasmic bridges connecting round spermatids under phase contrast optics and recorded the sharing of cytoplasmic material between the spermatids by a digital time-lapse imaging system ex vivo. Only 28% of those entering the bridge were actually transported into other cell. Haploid cell specific monoclonal antibody TRA54 was localized in the granules migrating through the cytoplasmic bridge. Translation of antigenic epitope (sugar moiety) of TRA54 starts at early spermiogenesis and it localizes to the Golgi complexes of step 1 – 3 spermatids. TRA54 accumulate into acrosomic system in step 3-5 spermatids and in the elongated spermatids TRA54 was localized at the edge of the acrosomic system. The male germ cell specific organelle, the chromatoid body, was also seen to move through the intercellular bridge between neighbor spermatids. We were able to demonstrate that microtubule inhibitors prevented all organelle movements through the bridges and caused a disintegration of the chromatoid body. Finally, we also demonstrate that Golgi complex has a novel function during spermiogenesis by sorting the granules containing haploid cell specific gene product, TRA54, and is needed not only for formation of one acrosomic system but possibly also acrosomic systems in several spermatids.

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