Expression and physiological significance of murine homologues of Drosophila Gustavus

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Abstract

Understanding the genetic control of gametogenesis is a central goal of developmental biology and is important for treating infertility in humans. An approach to identifying critical genes in mammals is to search for and study homologues of genes known to play key roles in other organisms. In the fly, *Drosophila melanogaster*, GUS protein is a component of nuage, an electron-dense aggregation in early germ cells, and is required for oocyte development. GUS physically interacts with VASA, an RNA helicase thought to regulate mRNA metabolism. I identified two murine genes, *SSB-1* and *SSB-4*, that are similar to and likely homologues of *gus*. SSB-1, SSB-4 and GUS each contain two conserved regions, termed the SPRY domain and the SOCS box, respectively. SSB-1 and SSB-4 share about 75% sequence identity and about 70% identity with GUS. Both SSB-1 and SSB-4 RNA and protein were found to be express in mouse ovarian granulosa cells of all stages of folliculogenesis. These cells support oocyte development and also produce steroids. Unexpectedly, SSB-1 and SSB-4 were only weakly or not detectable in oocytes, that contrasts with the expression of GUS in *Drosophila* oocytes. However, SSB-1 mRNA and protein were expressed in male germ cells; specifically in spermatocytes and spermatids. SSB-1 in spermatids was localized in a specialized structure known as the chromatoid body. Although the function of this structure is not quite clear, it has been compared to nuage, and one of its components is MVH, the murine homologue of VASA. Finally, using RNAi technology, SSB-1 was transiently depleted SSB-1 from a granulosa cell line. These cells showed a transient decrease in expression of the gene encoding P450scc, the rate-limiting enzyme in steroid synthesis. Preliminary results also indicated a decrease in progesterone synthesis. Taken together, these results establish the

expression pattern of murine homologues of *Drosophila* GUS in mouse ovary and testis, reveal it might play function in translation regulation in male spermatogenesis, and identify a potential role in steroidogenesis by ovarian granulosa cells.

Résumé

Une meilleure compréhension des mécanismes moléculaires impliqués dans la gamétogenèse est essentielle au développement d'outils thérapeutiques visant le traitement de l'infertilité chez l'humain. Puisque plusieurs gènes qui régissent la gamétogenèse sont conservés à travers les espèces, j'ai utilisé une approche d'homologie de séquences afin d'identifier des gènes de mammifères homologues au gène codant pour la protéine GUS. Chez la drosophile, GUS fait partie de « nuage », une agrégation électronique dense présente dans les cellules germinales précoces, et est requis pour le développement ovocytaire. GUS se lie à VASA, une ARN hélicase impliquée dans le métabolisme des ARN messagers. Ma recherche par homologie de séquence dans le génome murin a conduit à l'identification de deux gènes qui possèdent un important degré d'homologie avec *gus* : *SSB-1* et *SSB-4*. SSB-1, SSB-4 et GUS contiennent deux régions fortement conservées, un domaine SPRY et une boîte SOCS. L'identité de séquence entre SSB-1 et SSB-4 est de 75% et d'environ 70% avec GUS. J'ai démontré que SSB-1 et SSB-4 sont tous deux exprimés dans les cellules de la granulosa au cours de la folliculogenèse. Les cellules de la granulosa sont essentielles pour la maturation ovocytaire et pour la production d'hormones stéroïdiennes. Contrairement à une expression de GUS dans l'ovocyte chez la drosophile, l'expression de SSB-1 et SSB-4 est très faible ou absente dans l'ovocyte murin. Chez la souris, SSB-1 est aussi exprimé dans les cellules germinales mâles, plus précisément dans les spermatocytes et les spermatides. Au niveau des spermatides, l'expression de SSB-1 est restreinte à une structure spécialisée connue sous le nom de corps chromatoïde. Bien que la fonction exacte du corps chromatoïde ne soit pas bien caractérisée, elle semble similaire à la fonction

de «nuage», et l'une de ces composantes est MVH, l'homologue murin de VASA. Finalement, j'ai utilisé une approche d'interférence ARN afin de dépléter SSB-1 dans une lignée de cellules de la granulosa. Ces cellules montrent une réduction de l'expression du gène codant pour p450ssc, un enzyme limitant de la stéroïdogenèse. Des résultats préliminaires indiquent une diminution concomitante de la synthèse de progestérone. Les résultats obtenus au cours de cette étude ont donc permis d'établir le patron d'expression ovarien et testiculaire de deux homologues murins du gène gus et d'identifier des rôles potentiels pour ces facteurs aux niveaux de la spermatogenèse et de la stéroïdogenèse ovarienne prenant dans les cellules de la granulosa.

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Abbreviations

ABP	Androgen-binding protein
AMH	Anti-müllerian hormone
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
COC	Cumulus-oocyte complex
CPP	Cell-penetrating peptide
CXs	Connexins
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EST	Expressed sequence tag
FIGα	Factor in the germline α
FSH	Follicle simulating hormone
GCS	granulocyte colony-stimulating factor
GDF9	Growth differentiation factor 9
GOC	Granulosa cell-oocyte complex
HGL	germ cell less
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
IFN-γ	Gamma interferon
JNK	c-Jun terminal kinase

KL	kit ligand
LH	Luteinizing hormone
MEM	Eagle's Minimum Essential Medium
MTR-1	Mouse tudor repeat-1
MVH	Mouse VASA homologue
PGC	Primordial germ cells
RNAi	RNA interference
RyR	ryanodine receptor
siRNA	small interfering RNA
SnRNP	Small nuclear ribonucleoprotein
SRE	Serum response element
SOCS	suppressor of cytokine signalling
SSB	SPRY domain SOCS box
TGFβ	Transforming Growth Factor beta
UTR	untranslated region
VEGF	Vascular endothelial growth factor
XTR	*Xenopus* tudor repeat
ZP	Zona pellucida

Publications

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Contributions of Authors

All experiments described in this thesis were performed by Yan Xing except the following:

Prof. Roger Gosden and Prof. Paul Lasko provided intellectual contributions to the conceptual design of the research described in **Chapter 2**.

Chapter 3: Prof. Sarah Kimmins generated Figure 3. Sophie La Salle and Prof. Jacquetta M. Trasler provided the purified populations of male germ cells.

Chapter 4: Progesterone assay was performed in the laboratory of Prof. Bruce Murphy.

1. GENERAL INTRODUCTION

1.1 Mammalian ovary and stages of folliculogenesis

1.1.1 Anatomy of the mammalian ovary

The ovaries are part of the reproductive system in human. A woman normally has two ovaries and each of them serves two major functions. The first is a reproductive function – to produce eggs that are competent for fertilization and embryonic development. The second is an endocrine function – to produce steroid and peptide hormones.

When the mature human ovary is observed in the section (Figure 1), it consists of an outer zone – the cortex, and an inner zone – the medulla. The surface tissue is called the germinal or surface epithelium. The resting primordial follicles are located in the ovarian cortex. In contrast, the growing (primary, secondary and Graafian follicle) and atretic follicles, as well as the corpora lutea derived from follicles that have undergone ovulation, are found more interiorly. Ovarian stromal cells localize between follicles. Each follicle is surround by a hormone- producing layer of cells known as the theca cell. The ovary also contains dense connective tissue in the medulla and the stromal cells, blood vessels and lymphatics are embedded in it (Leung & Adashi 2004).

1.1.2 Stages of oogenesis and folliculogenesis

In many non-mammalian species, including the fruit fly, *Drosophila,* and the worm, *C. elegans*, the germ cells are derived from maternal components that are localized in specific regions of the egg and allocated to specific cells during the cleavage divisions of early embryogenesis (Wylie 1999). In contrast, in the mouse and probably all mammals, the germ cells are derived from the proximal epiblast, which also gives rise to extraembryonic mesoderm and other mesodermal tissues in the posterior region of the

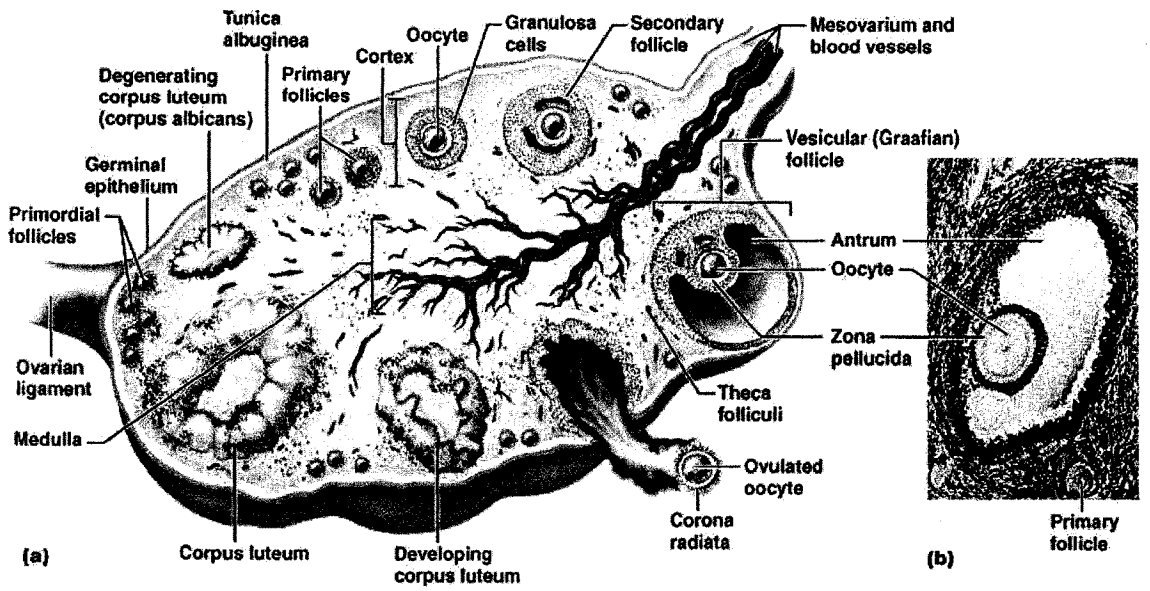


Figure 1: Anatomy of the human ovary and follicle

(A) Schematic representation of an adult human ovary showing the main follicular and luteal phases that can be observed in a histological section. In ovarian cortex, four stages of folliculogenesis are illustrated including: primordial follicle, primary follicle, secondary follicle and graafian follicle. The supporting granulosa cells and steroidogenic thecal cells are also shown. After ovulation, the remaining granulosa cells luteinize and form the corpus luteum. There are connective tissues and blood vessels in ovarian medulla. (B) A human ovarian section showing an antral and a primary follicle (250 X). Taken from Human Anatomy and Physiology, 6th Editon, Elaine N.Marieb, San Francisco : Pearson/Benjamin Cummings, ©2004.

host embryo (Tam & Zhou 1996b). In the mouse, the primordial germ cells first become detectable at about day 7.5 of development, shortly after gastrulation, owing to their high alkaline phosphatase activity. They subsequently migrate dorsally and laterally until they reach and colonize the primitive gonads lying adjacent to the kidney primordium. During this stage, they continue to proliferate. At about day 13.5, however, germ cell mitotic activity stops – female germ cells enter meiosis and male germ cells remain mitotically arrested until after birth. Meiosis proceeds as far as the diplotene stage, where it becomes arrested. The chromosomes are partially condensed and packed within the nucleus, which is known as the germinal vesicle.

The somatic tissue of the gonad develops from the mesenchymal cells. In females, these mesenchymal cells remain mingled with the germ cells until late embryonic life. Near the time of birth, however, the mesenchymal cells secrete an outer basement membrane and surround individual oocytes to form primordial follicles. At this stage, the somatic cells are named granulosa cells. Recent work indicates that the formation of primordial follicles is linked to the breakdown of cysts of germ cells, which previously were attached by intercellular bridges (Pepling & Spradling 2001). Thus at birth, the mammalian ovary contains thousands of primordial follicles, each containing a single oocyte surrounded by a small number of flattened granulosa cells.

Beginning at sexual maturity, small cohorts of primordial follicles regularly enter the growth phase and develop to fertilizable eggs. This process ensures that, even though no new oocytes are generated after birth, the female is provided with fertilizable eggs throughout reproductive life (Eggan *et al.* 2006). When the pool of primordial oocytes becomes exhausted, the female can no longer reproduce, and in humans this event

triggers menopause. Thus, in humans, the follicle may remain at the primordial stage for up to 50 years until a signal initiates oocyte and follicle growth (Smitz & Cortvrindt 2002).

The first sign that an oocyte has entered the growth pool is the transition of the granulosa cells from a flattened to a cuboidal shape (Pedersen & Peters 1968). These are now termed primary follicles. Other hallmarks of the primary follicle stage are the secretion by the oocyte of the zona pellucida, an extracellular coat that surrounds the oocyte and serves to prevent polyspermy and protect the egg following ovulation (O'Shaughnessy *et al.* 1997). As the oocyte continues to grow, the granulosa cells also proliferate, generating up to seven layers of cells. As the number of granulosa cells increases, fluid-filled spaces begin to appear between the granulosa cells; such follicles are termed early antral. These fluid-filled spaces then coalesce to produce a large antrum. When this antrum has grown very large owing to increased fluid production, just before ovulation, the follicle is termed 'Graafian.'

At preantral stages, the granulosa cells acquire high-affinity FSH and estrogen receptors and are a morphologically and functionally homogeneous population (Richards 1975; Richards *et al.* 1976). Once the fluid-filled spaces have become coalesced into a large cavity, however, the granulosa cells become separated into two populations: the mural cells, located at the periphery of the follicle, and the cumulus cells, which surround the oocyte (Smitz & Cortvrindt 2002). Importantly, morphological and functional differences exist between these cell populations. Mural granulosa cells adjacent to the basement membrane elongate, stop proliferating, synthesize enzymes involved in estrogen production, and acquire LH receptors. In contrast, the innermost layers of mural

granulosa cells, adjacent to the antrum, and the cumulus cells remain polyhedral, continue to proliferate, show limited steroidogenic activity, and acquire fewer LH receptors (Channing *et al.* 1981). In addition, these two cell populations produce different growth factors. For example, cumulus cells express higher mRNA levels of insulin-like growth factor (Channing *et al.* 1981) and VEGF (Dissen *et al.* 1994), whereas the mural granulosa cells express higher levels of the mRNA encoding Kit ligand (Manova *et al.* 1993).

The systemic surge of LH, released by the pituitary gland, induces the Graafian follicle into the final stage of development. Oocytes at this stage have acquired the competence to resume meiosis and to undergo fertilization and preimplantation development (Sorensen & Wassarman 1976). LH triggers the resumption of meiosis, a process termed meiotic maturation. During maturation, the oocyte is released from prophase I arrest, completes the first meiotic division during which either the maternal or the paternal homologue of each chromosome pair is discarded in the first polar body, and advances to metaphase of the second meiotic division (metaphase II), where it becomes arrested until fertilization. Since the LH receptors are localized on the mural granulosa cells, these must send a signal to the oocyte to trigger meiotic maturation. Recent work suggests that EGF-related peptides may be the LH-induced signal produced by the mural cells (Park *et al.* 2004), though whether these act directly on the oocyte or via the cumulus granulosa remains unknown.

Meanwhile, LH also triggers synthesis by the cumulus cells of a mucoelastic extracellular matrix made up mainly of hyaluronic acid. The formation of this matrix between the cumulus cells causes them to separate from each other and also from the oocyte, and the

entire cumulus-oocyte complex (COC) becomes larger; hence, this process is often termed cumulus cell expansion or mucification. Cumulus expansion will help the COC detach from the follicle wall (Salustri *et al.* 1992). Under the influence of LH, the mural granulosa cells start to synthesize progesterone, which can induce the expression of proteolytic enzymes, leading to the degradation of the perifollicular matrix by an autocrine pathway (Richards *et al.* 2002). Consequently, the follicle wall breaks and COC is extruded from the ovary.

Following ovulation, the cumulus cells and their mucoelastic extracellular matrix provide the proper environment for the oocyte successful fertilization. In the ovary, the mural granulosa cells together with the theca cells form the corpus luteum, which produces the high level of progesterone required for successful implantation of the embryo and maintenance of the pregnancy (el-Fouly *et al.* 1970). In the rodent, the corpus luteum is required throughout pregnancy, whereas in humans the placenta takes over progesterone production by about the fourth month of gestation.

1.2 Genetic control of folliculogenesis in mice

Many genes take part in the genetic regulation of the embryonic development of the ovary and the tight control of the different stages of folliculogenesis. The reproductive organs, ovary and testis are likely to express more genes than the other organs because of the presence of both somatic and germ cell types. It has been found that there were 21,000 known genes and ESTs and 6,000 unknown tags expressed in human oocytes (Neilson *et al.* 2000).

The formation of primordial follicles depends on the close interaction between oocytes and pre-granulosa cells. During the following steps of folliculogenesis, producing an oocyte that is competent to undergo fertilization and forming a corpus luteum that sustains the development of the embryo also rely on the intimate cooperation of oocytes and somatic cells. As discussed above, gonadotropins produced by the pituitary gland have a critical role in the regulation of these processes. But the initial stages of folliculogenesis, from primordial to early antral follicles, are independent of gondadotrophins and their regulation is still poorly understood at a molecular level. Analysis of the phenotypes of 'knock-out' mice lacking specific genes has elucidated the function of these genes in this process and confirmed that successful oogenesis depends on genetic networks linking germ cells and somatic cells (Epifano & Dean 2002; Choi & Rajkovic 2006; Roy & Matzuk 2006).

1.2.1 Formation of primordial follicles

As noted above, the primordial germ cells (PGC) are specified from a pool of proximal epiblast cells at around embryonic day-7.5 (Roy & Matzuk 2006). BMP signalling appears to be necessary for PGC fate specification. In BMP4-null mutant mice, PGCs are completely absent (Lawson *et al.* 1999). Knock-out mice lacking other transforming growth factor β (TGFβ) superfamily members, BMP8b and BMP2, showed very similar phenotypes of reduced or absent PGCs (Ying *et al.* 2000; Ying & Zhao 2001). Knockout mice lacking the serine/threonine kinase receptor ActRIA (ALK2), the type I BMP receptor, were also found to have a complete absence of PGCs, further highlighting the importance of BMP signaling pathway in germ-cell specification (de Sousa Lopes *et al.* 2004). The detailed mechanism of this signalling pathway is still elusive.

A recently found mouse zinc-finger containing transcriptional repressor, PRDM1 (PR domain-containing 1, with zinc-finger domain) also plays an essential role in PGC specification. *Prdm1* is expressed in PGCs as early as embryonic day-7.0. *Prdm1* homozygous mutant embryos entirely lack PGCs and the number of PGCs in heterozygous embryo is also significantly reduced compare to wild type. This is the first transcriptional factor found to function in PGC specification (Vincent *et al.* 2005).

FIGα (factor in the germline α), an oocyte specific transcription factor, is expressed from as early as embryonic day-13. In mutant mice lacking *FIGα*, the number of oocytes is similar to wild-type until embryonic day-19. However, at postnatal day-1, the oocytes begin to disappear. By postnatal day-2, the mutant mice have completely lost the primordial follicles and the scattered remaining oocytes completely disappear within 7 days, the ovaries shrink in size, and the females are sterile. *FIGα* null mutant mice do not

express zona pellucida gene *ZP1, ZP2, ZP3*. This phenotype suggests that oocyte-specific *FIGα* is required for the initial formation of primordial follicles and also for the expression of other important genes that function in oocyte survival (Soyal *et al.* 2000).

Wnt-4 knock-out mice show a similar phenotype. *Wnt-4* is expressed in somatic cells and the phenotype of *Wnt-4* knock-outs implies that the formation of primordial follicle needs a somatic factor. *Wnt-4* is a signalling molecule that is expressed in the mesonephric mesenchyme of the indifferent gonad in both sexes, where the gonadal somatic lineage is derived (McLaren 1991). *Wnt-4* null mutants only have 10% of the oocytes as compared to wild type mice during the process of masculinization of the female gonad and even these oocytes are in the process of degenerating. Primordial follicles are rarely found in *Wnt-4* null mutant, and some granulosa cells show partial transdifferentiation into Sertoli cells. Male development is unaffected by the loss of *Wnt-4* (Vainio *et al.* 1999). While *FIGα* is expressed in germ cells and *Wnt-4* is expressed in somatic cells, the similarity of phenotype between the null mutants proves that interactions between oocytes and granulosa cells are important from the time of primordial follicle formation.

1.2.2 Initiation of oocyte and follicle growth

The mechanism that triggers growth of selected follicles (i.e., transition from primordial to primary follicle) in the ovary remains to be determined. However, several genes involved in early stages of growth have been identified. *Nobox* is a recently identified oocyte-specific homeobox gene that plays a crucial role in the primordial to primary transition. NOBOX protein is expressed in the germ cell cysts, primordial and growing oocytes throughout different stages of folliculogenesis (Suzumori *et al.* 2002). To make

the null mutant mice, 90% of the *Nobox* coding region was deleted including exons that encode the homeodomain. Using two germ cell markers, GCNA1 (germ cell nuclear antigen) and MSY2 (germ cell–specific Y box protein, a cytoplasmic marker for oocytes), the results of immunohistology indicated that the relative number of oocytes, germ cell cysts and primordial follicles were normal in *Nobox* mutant mice compared to wild type at birth, implying no germ cell loss before birth. At postnatal day-3, however, few primary follicles were found in homozygous null mice compared to heterozygous mice and wild type. In contrast, homozygous mice contained more germ cell cysts and primordial follicles than heterozygous and wild type mice. At postnatal day-7, secondary follicles were obvious in the heterozygous but were not found in homozygous mice. At postnatal day-14, very few oocytes were present in homozygous mice. These observations indicate that NOBOX functions in activating the transition from primordial to primary follicle. In addition, in the *Nobox* null mutant, several genes which are specifically expressed in oocytes and play important functions in oogenesis and early embryogenesis, including *GDF9, BMP15, ZAR1* and *Dnmt1,* are not expressed. However, the expression of other genes, which are not specifically expressed in the oocyte but function in early folliculogenesis, such as *Bmp4, and Wnt4*, remains unchanged. Therefore, *Nobox* appears to regulate genes which are specifically expressed during oognesis (Rajkovic *et al.* 2004). Early oocyte growth also depends on the interaction of KIT, a tyrosine kinase receptor expressed in oocytes, and Kit ligand (KL), which is produced by the granulosa cells. Mice with naturally occurring mutations in the gene encoding KL can form primordial follicles and progress to primary stage but do not form multi-layered follicles (Huang *et al.* 1993). In addition, KL can accelerate the growth of the cultured denuded oocytes isolated from

preantral ovaries (Klinger & De 2002). On the other hand, an anti-KL antibody, ACK2, did not affect the formation of primordial follicle but blocked the transition of primordial to primary follicle (Yoshida *et al.* 1997). Consistent with these results, an inhibitor of tyrosine kinase prevents the growth of oocytes when added to cultured ovarian follicles (Packer *et al.* 1994; Yoshida *et al.* 1997). These results indicate a key role for KL-Kit signalling at different stages of oocyte growth.

1.2.3 Progression of follicle growth

The dominant role of the oocyte in regulating the progression of follicular development has been elegantly demonstrated by Eppig's group (Eppig *et al.* 2002). Mid-growth-phase oocytes from 12-day old mice were isolated and reaggregated *in vitro* with granulosa cells isolated from primordial follicles of newborn mice. The reconstituted follicle was then implanted beneath the renal capsule. It developed into the antral stage, nine days after implantation, instead of 21 days after implantation. This result demonstrates that the oocyte controls the proliferation and differentiation rate of the follicle according to its intrinsic developmental stage.

Nonetheless, the granulosa cells also play essential roles in enabling oocyte growth. Bidirectional communication between the oocyte and the surrounding granulosa cells is critical for oocyte development. For example, oocytes isolated at mid-growth stages can not grow significantly *in vitro* without physical association with granulosa cells (Klinger & De 2002).

Oocyte development depends on gap junctions that link the oocyte to the granulosa cells and the granulosa cells to each other. Gap junctions are channels, composed of proteins

known as connexins, which span the cell membranes and link neighbouring cells. They allow ions and small molecules (molecular weight less than 1000 daltons) to pass between cells. Thus, the communicating cells may exchange small molecules (e.g., Ca^{2+}, cyclic AMP, glutathione) as well as macromolecular components (e.g., nucleotides, amino acids; sugars). However, different connexins are expressed in different cell types, suggesting that specific properties of a gap junction may be determined by the type of connexin that forms it (Kidder & Mhawi 2002) (Figure 2).

Connexin-43 is the most abundant protein in the gap junctions that link cumulus cells. Mice lacking Cx43 as a result of a targeted disruption of *Gja1* die soon after birth, which is the result of a severe heart abnormality (Reaume *et al.* 1995). Homozygous gonads of both sexes are unusually small and have only 10% of the germ cell population of wild type mice. Since the null mutant is neonatal lethal, experimental methods were used to investigate the folliculogenesis in Cx43 knock-out mice. When neonatal ovaries were cultured in vitro, postnatal folliculogenesis was retarded (Juneja *et al.* 1999). Neonatal ovaries were then grafted into the kidney capsules of ovariectomized, immunocompromised adult mice to permit postnatal development for three weeks. Grafted wild-type ovaries developed follicles from primordial to antral stages; however, the mutant mice failed to develop to multilayered follicles (Ackert *et al.* 2001). These results imply that Cx43 is necessary for granulosa cell proliferation and plays an essential role in folliculogenesis.

Another gap junction protein, connexin-37 (Cx37), is the only gap junction protein known to be contributed by oocyte. Null mutant mice for Cx37 generated by a targeted disruption of the *Gja4* gene are viable and ovarian folliculogenesis is normal up to the

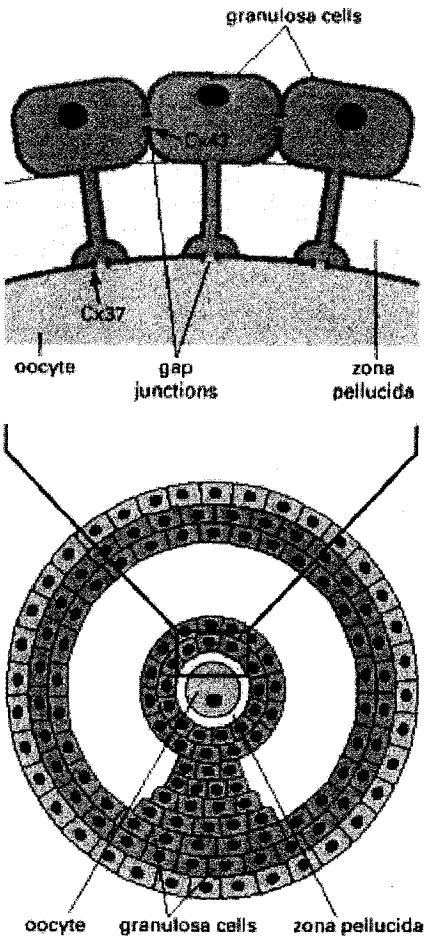


Figure 2: Gap junction coupling in the ovarian follicle.

Schematic representation of gap junctions that link the oocyte to the granulosa cells and the granulosa cells to each other. Gap junctions are channels, composed of proteins known as connexins which span the cell membranes and link neighbouring cells. The communicating cells may exchange components (e.g., Ca^{2+}, cyclic AMP, nucleotides, amino acids; sugars) through gap junctions. Connexin43 (Cx43) is the gap junction linking cumulus cells and Connexin37 (Cx37) is the only gap junction contributed by oocyte. Taken from Kidder& Mhawi 2002.

antral follicle stage (Simon *et al.* 1997). There are no Graafian follicles in these mice, however, and ovulation cannot be induced by gonadotrophin stimulation. There are numerous structures resembling small corpora lutea in null mutant ovary. These results suggest the Cx37 prevents the granulosa cells from differentiating prematurely before fertilization (Carabatsos *et al.* 2000).

A recent publication provides further evidence for granulosa cell-oocyte interaction, by showing that the granulosa cells regulate the intracellular pH of the growing oocyte. It was previously found that denuded (i.e. without the granulosa cells) growing oocytes have 0.25 unit lower pH value than do fully grown oocytes (6.95 compared to 7.2). The denuded growing oocytes are not able to regulate the intracellular pH (pH_i) and the pH_i-regulatory HCO_3^-/Cl^- and Na^+/H^+ exchange activities are not active (Erdogan *et al.* 2005). However, in this paper, it was found that in the intact follicle (oocyte-cumulus complex), the oocyte pH_i remains 7.2 throughout oocyte development and that the growing oocyte in the complex has the ability to carry out HCO_3^-/Cl^- exchange. Interestingly, this exchange can be blocked by gap junction inhibitors. These results imply that the pH_i of growing oocytes is controlled by granulosa cells through gap junctions. Thus, the oocyte did not gain the ability to maintain its own homeostasis until a certain developmental stage (Fitzharris & Baltz 2006).

The initial stages of folliculogenesis are independent of pituitary gonadotropins and mainly regulated by intraovarian paracrine factors. So far, three members of transforming growth factor-β family – anti-Müllerian hormone (AMH), BMP-15 and GDF-9 – have been found to regulate early folliculogenesis. AMH is expressed in granulosa cells and appears to function in recruiting primordial follicles into folliculogenesis (Durlinger *et al.*

1999). In females, AMH is produced by the ovary postnatally. Female null-mutant mice are fertile with normal litter size. However, when the complete follicle population was counted at 25 days, 4 months and 13 months of age, AMH null mutants contained more preantral and antral follicles but fewer primordial follicles than wild-type mice at 25 days and 4 months. At 13 months, almost no primordial follicle could be detected in the mutants. Thus, AMH null females show early depletion of primordial follicles, implying that AMH plays an important role in recruitment of primordial follicles.

GDF-9 and BMP15 are specifically expressed in oocytes. They are first detectable in the primary follicle stage and persist until after ovulation (McGrath *et al.* 1995; Dube *et al.* 1998). In GDF-9 null mutant mice, folliculogenesis cannot proceed beyond the primary stage and the females are sterile (Dong *et al.* 1996). Mice lacking BMP-15 are subfertile with decreased ovulation and fertilization rate but normal ovarian histology (Otsuka *et al.* 2000). However, naturally occurring mutations of the homologous gene in sheep, termed *FecX*, causes arrest of follicular development at the primary stage (Galloway *et al.* 2000), similar to the phenotype observed in GDF-9 null mice. In all three cases (absence of AMH, GDF-9 and BMP-15), primordial follicles appear normal, which suggests that they do not regulate this early developmental stage (Kissel *et al.* 2000).

1.2.4 Development of antral follicles and ovulation

Follicle development from the primordial to the early antral stage is controlled mainly by intraovarian factors. Conversely, two hormones secreted by pituitary gland, FSH and LH, play essential roles in the growth of antral follicles and acquisition of the ability to

respond to ovulatory stimulation (Kumar et al. 1997). The systemic surge of FSH and LH induces the large antral follicle to enter into the final stage of development. At antral follicles stages, oocytes acquires full competence to complete meiotic maturation and preimplantation embryo development (Eppig et al. 1996). Oocytes spontaneously resume meiosis when they are isolated from antral follicles and cultured in vitro (Edwards 1965); however, oocytes do not resume meiosis when cultured as complexes of granulosa cells and oocytes (Tsafriri & Channing 1975). Therefore, premature meiotic resumption is prevented by the follicular environment.

The acquisition of LH receptors by granulosa cells takes place during the antral stage and is an essential step in progression to the final phase of ovulation and luteinization (Oxberry & Greenwald 1982). The expression of LH receptors shows a gradient in the antral follicle. They are abundant in the outermost layers of mural granulosa cells, and show a steadily decreasing abundance towards the middle of the follicle, becoming undetectable in the cumulus cells which surround the oocytes (Eppig 1979). The reason for the graded distribution of LH receptors is not understood, but the oocyte is known to play a dominant role in establishing it. Experimental evidence has shown that when the oocyte is removed from isolated cumulus-oocyte complexes, LH receptors will appear on the cumulus cells, and the increased expression is suppressed when the cumulus cells are cultured in oocyte-conditioned medium. On the other hand, the expression of LH receptor in mural granulosa cells decreases dramatically when they are co-cultured with oocytes (Eppig *et al.* 1997). Thus, it appears that oocytes produce a soluble factor that inhibits the expression of LH receptors by granulosa cells.

Although the mechanism by which LH induces the events of ovulation remain to be clarified, it is interesting that, *in vitro*, GDF-9 induces mouse cumulus expansion and up-regulates the expression of genes known to be involved in cumulus matrix formation and stability (Elvin *et al.* 1999). This suggests that GDF-9 or a similar molecule may be up-regulated by LH during the ovulation process.

Overall, the different stages of folliculogenesis depend on a strict genetic control and intimate coordination between germ cells and somatic cells. Although only a few gene products involved in folliculogenesis have been identified, there is no doubt that more and more gene candidates will be added to this list (Table 1) with the development of mouse genetics. Detailed understanding of the molecules involved in the germ and somatic interactions will help us elucidate the mechanism of mammalian fertility.

Table 1: Mouse genes affecting early folliculogenesis

Gene	Phenotype of transgenic or knockout mouse	References
Figla	knockout; infertile; oocyte loss by postnatal day 2; unable to form primordial follicles	Soyal *et al.*,2000
Nobox	knockout; infertile; most oocytes lost by postnatal day 14; primordial to primary follicles transition disrupted	Rajkovic *et al.*,2004
Sl ^{d}	spontaneous mutation; infertile; lack of germ cells	Brannan *et al.*,1991
Sl $^{t, pan, con}$	spontaneous mutation; infertile; defect in folliculogenesis at primordial follicle stages; reduce number of germ cells	Bedell *et al.*,1995 Huang *et al.*,1993 Kuroda *et al.*,1988
MT-Amh	Amh transgenic overexpression; infertile; lack of Müllerian duct derivatives; rapid loss of germ cells by postnatal day 14	Behringer *et al.*,1990
Foxl2	knockout; infertile; block at stage of the primordial and primary follicle	Schmidt *et al.*,2004 Uda M *et al.*,2003
Ngf	knockout; reduced number of primary and secondary follicles; reduced proliferation of somatic cells	Dissen *et al.*,2001 Ojeda *et al.*,2000
Ntf4/5&Bdnf	double knockout; reduced number of primary and secondary follicles	Liebl *et al.*, 2000 Paredes *et al.*, 2004
Ntrk2	knockout; failed transition from primary to secondary follicle	Paredes *et al.*, 2004
Gdf9	knockout; infertile; arrest of follicle growth at the primary follicle stage	Dong *et al.*, 1996
Bmp15	knockout; subfertile; defects in ovulation and fertilization	Yan *et al.*, 2001

Taken from Choi&Rajkovic 2006.

1.3 Methods of follicle culture

Progress in understanding the mechanisms that regulate folliculogenesis has been greatly facilitated by the development of culture systems that support this process. Culture systems are most advanced for the mouse, which is the only mammal from which a fertilized egg and live offspring have been achieved from primordial follicles developing entirely *in vitro* (Eppig & O'Brien 1996). Several methods have been developed for folliculogenesis *in vitro*.

The system developed by Eppig uses partially grown oocytes recovered from preantral follicles of 12-day old mice. The oocytes are surround by relatively undifferentiated granulosa and thecal cells. The granulosa-oocyte complex can be recovered by collagenase digestion, which removes the thecal and stromal cells. The granulosa cell-oocyte complexes grow on a collagen-impregnated membrane at moderate density (200/per well of 6-well collagen insert), which enables them to attach without much spreading, in a defined serum-free medium that contains bovine serum albumin (BSA), insulin, transferrin, selenium, and hypoxanthine. During culture, the complexes often extend a stalk of granulosa cells that bears an oocyte, reminiscent of the structure of a Graafian follicle (Figure 3a). Oocytes cultured in this system will be fully grown after 10 days of culture (Eppig *et al.* 1992). In this culture system, the intimate interaction between oocytes and granulosa cells is preserved, which is important for oocyte growth and development (Gosden *et al.* 2002). However, it does not reproduce follicular development, as the thecal cells are absent. For example, LH receptors do not appear on the granulosa cells, likely due to the inhibitory influence of the oocyte (see above).

Other methods of follicle culture are shown in Figure 3. One involves placing the follicle within of unhealthy follicles (Torrance *et al.* 1989). The simplest method is to place intact follicles on a plastic substrate in the presence of serum or BSA (Figure 3c). In this culture system, both granulosa and thecal cells remain present and their spatial arrangement with respect to each other and the oocyte is maintained (Cortvrindt *et al.* 1996). However, the entire structure tends to flatten onto the dish, producing a pancake-shaped oocyte of uncertain developmental potential. Finally, some researchers grow the follicles in microdrops of medium under mineral oil (Figure 3d). Using these conditions, 80% of follicles ovulated *in vitro* in response to hCG (Rose *et al.* 1999). In the work described in this thesis, collagen-impregnated membranes and plastic culture dishes were used to investigate SSB-1 function using RNA silencing.

1.4 Anatomy of the testis and stages of spermatogenesis

The mature mammalian testis consists of a dense network of seminiferous tubules lying within a matrix of supporting cells. The seminiferous tubules consist of the germ cells, the Sertoli cells, and the peritubular myoid cells. The extratubular tissue consists of the Leydig cells, blood vessels, lymphatic fluid and fibroblasts (Nussey S.S & Whitehead S.A 2001). (Figure 4)

The process of male gamete production is called spermatogenesis, and this process takes place in the seminiferous tubules. During embryogenesis of males, the primordial germ cells upon reaching the gonad continue to proliferate to generate spermatogonia. Spermatogonia are stem cells, in that mitotic division produces a new cell capable of division and a sister cell that will begin the process of spermatogenesis. Differentiating spermatogonia actually undergo several more mitotic divisions, during which they are termed type A, intermediate, and type B spermatogonia. Type B spermatogonia are the final product of the mitotic phase of spermatogenesis. They are the precursors of spermatocytes, which undergo two cycles of meiotic division. Meiotic division produces haploid round spermatids. During the process of spermiogenesis, these undergo extensive morphological and molecular remodelling, including the loss of most of their cytoplasmic components, to produce mature sperm.

The combination of mitotic and meiotic divisions undergone by a type A spermatogonium means that each division of the spermatogonial stem cell can result in the production of over one hundred mature sperm (Figure 5). The cytokinetic divisions that occur during this process of clonal expansion are incomplete, meaning that all products remain

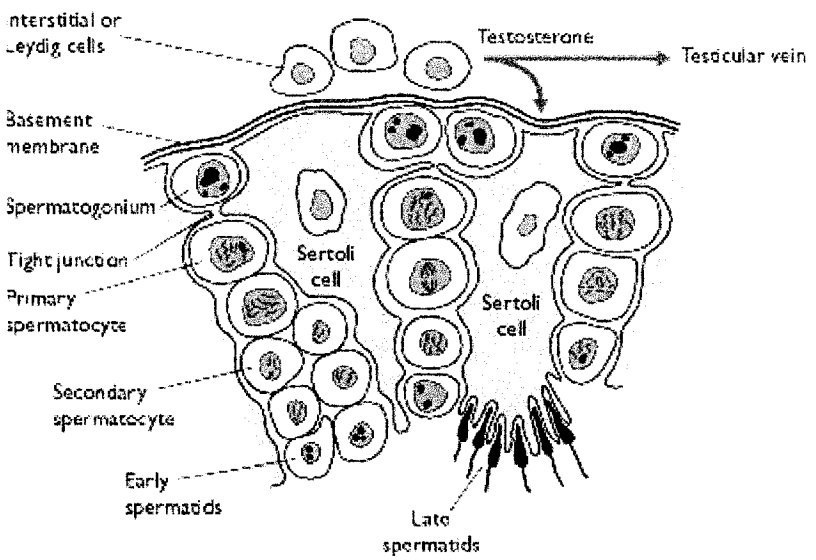


Figure 4: Anatomy of the human testis

Schematic representation of a section of the human seminiferous tubule, showing the germ cells, Sertoli cells and Leydig cells that can be observed in a histological section. Germ cells from spermatogonium to spermatids are localized in the seminiferous tubule and, as the cells mature, they progress toward the lumen of the seminiferous tubule. Sertoli cells are located on the basement membrane but their apical surface is exposed to lumen. Sertoli cells supply nutrients and growth factors and function to support and regulate spermatogenesis. Leydig cells are located in the extratubular space of the testis and produce testosterone. Taken from Endocrinology, an integrated approach, S.S. Nussey and S.A.Whitehead, London: Taylor&Francis, ©2001.

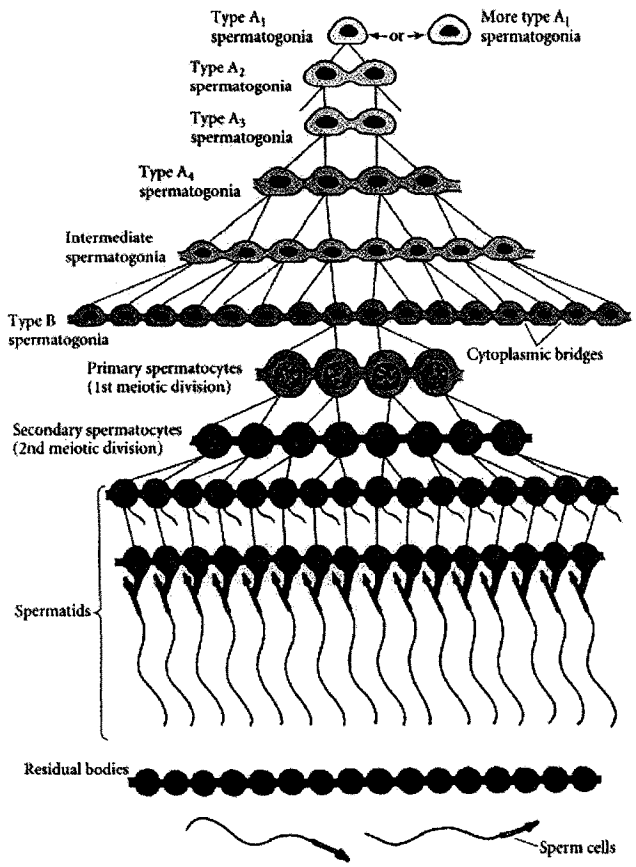


Figure 5. Different stages of spermatogenesis in human male germ cells.

Once primordial germ cells reach the gonad in early embryogenesis, they divide to produce spermatogonia. Spermatogonia undergo mitotic divisions to generate type B spermatogonia, the precursors of spermatocytes. The primary spermatocytes undergo the1st meiotic division to become secondary spermatocytes. After completing the 2nd meiotic division, the secondary spermatocytes produce haploid spermatids, which differentiate into mature sperm. Taken from Developmental biology, 6th edition, Scott F. Gilbert, Sunderland, Mass: Sinauer Associates, Inc., ©2000

connected by intercellular bridges through which large molecules including proteins can pass. Thus, even though spermatids will carry different genomes, their products may be shared. The cytoplasmic bridges are broken and possibly absorbed by Sertoli cells when sperm cells are released into the lumen of the tubules.

Figure 5 also illustrates another unique characteristic of spermatogenesis; namely, the luminal progression of the cells during differentiation. Spermatogonia are found at the basement membrane, where they are embedded in cytoplasmic extensions of the Sertoli cells. During the meiotic divisions, the spermatocytes migrate towards the lumen while retaining their association with the Sertoli cells. Spermatids are found luminal to the spermatocytes, while the mature sperm, upon release from the Sertoli cells, collect in the lumen from which they will be transported to the epididymis and vas deferens. Thus, the position of the developing male germ cell in the tubule provides information about its state of developmental progression (Gilbert 2000).

As noted above, the spermatogenic cells are intimately associated with the Sertoli cells. The Sertoli cells are terminally differentiated diploid somatic cells and are essential for spermatogenesis. Because the blood vessels do not enter the seminiferous tubules, Sertoli cells supply nutrients and growth factors, and eliminate metabolites from the tubules. Sertoli cells are also the mediator of various hormonal and growth factor actions to support and regulate spermatogenesis. For example, testosterone produced by Leydig cells diffuses from the interstitium to the seminiferous epithelium and is converted to the more active androgen, dihydrotestosterone, by 5α-reductase activity in the Sertoli cells. Sertoli cells produce the androgen-binding protein (ABP) that binds dihydrotesterosterone

and carries the hormone in the testicular fluid, flowing out into the blood circulation system and delivering the hormone to all around the body (Hansson *et al.* 1975).

The Sertoli cells also produce the peptide hormones, inhibin and activin (de Kretser *et al.* 2004) and, in addition to their spermatogenic role, function very early in embryogenesis by inducing differentiation of the bipotential gonad into a testis (Grootegoed *et al.* 2000).

The peritubular myoid cells are a smooth muscle type and, as their name suggests, surround the outer surface of seminiferous tubules. Their functions are not well understood, but they are believed to aid in contraction of the tubules, which could transfer sperm from the testis to the epididymis. Between the seminiferous epithelium and myoid cells is the basement membrane, made up largely of collagen-IV, fibronectin, and laminin (Nevstrueva & Boronikhina 1980).

The extra-tubular matrix of testis contains, in addition to blood vessels, the Leydig cells. Their chief function of Ledig cell is to produce testosterone, which is required for maintenance of spermatogenesis and induction of the male phenotype (Haider 2004).

1.5 Drosophila as a model for studying vertebrate development

1.5.1 Genes regulating oogenesis in Drosophila

During oogenesis in *Drosophila*, some mRNAs are translationally repressed and remain stored until fertilization. In addition, specific localization of mRNAs during oogenesis is essential for correct anterior-posterior axis formation. *Drosophila* nanos (*nos*) is a gene that encodes an RNA-binding protein (Wang & Lehmann 1991). It is classified as a maternal-effect gene because, in the absence of maternal *nos*, primordial germ cells (PGCs) can not migrate into the gonad and become functional germ cells (Forbes & Lehmann 1998). Three homologues of *nos* have been identified in mice. Genetic disruption of *nanos1* does not affect germ cell development (Haraguchi *et al.* 2003). The expression of *nanos2* is predominantly in male germ cells, and null mutants show complete loss of spermatogonia. In contrast, in female null-mutant mice, ovaries are morphologically normal and the animals are fertile. *Nanos3* is expressed in migrating primordial germ cells and undifferentiated gonads in embryos. In its absence, primordial germ cells are lost from both males and females during early embryogenesis, apparently even before reaching the primitive gonad. Hence, the null mutation causes complete loss of germ cells in both sexes.

Nanos protein associates with Pumilio (Forbes & Lehmann 1998) and maintains germ cell development through a translational repression mechanism. *Xcat-2*, a *nanos* homolog in frogs, localizes to germ plasm in that organism (Mosquera *et al.* 1993) and human NANOS-1 has recently been shown to be expressed in spermatogonia and to interact with human PUMILIO-2 (Moore *et al.* 2003; Jaruzelska *et al.* 2003). In summary, these results

suggest that function of NANOS proteins in germ cell development has been evolutionarily conserved among invertebrates and vertebrates (Tsuda *et al.* 2003).

The *staufen* gene of *Drosophila* encodes an RNA-binding protein that is required for the localization of maternal mRNA within oocytes (StJohnston *et al.* 1991). *Staufen* is essential to localize *bicoid* mRNA to the anterior of the oocyte and *oskar* mRNA to the posterior of oocyte correctly (StJohnston *et al.* 1991; Kim-Ha *et al.* 1991). In the mouse, RT-PCR and Northern blotting indicates that mouse *staufen* is expressed in both oocytes and testis. Results of *in situ* hybridization reveal that mouse *staufen* mRNA is expressed in mouse oocytes from the primary until antral follicle stage. In the testis, *staufen* mRNA is localized predominantly in the primary spermatocytes, decreasing in secondary spermatocytes and disappearing in differentiated sperm cells. Therefore, although *Drosophila staufen* plays a function only during oogenesis, the expression pattern of mouse *staufen* suggests possible roles in both oogenesis and spermatogenesis in rodents (Saunders *et al.* 2000).

One gene with a potential role in human fertility is *germ cell-less* (*gcl*). The GCL protein was first described in *Drosophila* as a crucial factor in embryonic germ cell development (Jongens *et al.* 1992). The mouse homologue of germ cell-less (*mgcl-1*) shows 34% identity compared with the *Drosophila* gene. It is expressed in low levels in different adult tissues and during germ cell specification and migration in mice, but at a very high level in adult testis within spermatocytes. The expression of *mgcl-1* in fly null-mutants of *gcl* can rescue germ cell formation, implying a functional conservation (Leatherman *et al.*

2000; Kimura *et al.* 1999). Further studies including gene disruption analysis would provide an important insight into its role during mammalian germ cell development.

Drosophila Tudor is essential for pole cell specification, polar granule formation and oogenesis (Thomson & Lasko 2004). Using the *Xenopus* Tudor Repeat (XTR) amino acid sequence (Ikema *et al.* 2002) as a query, a short EST was identified in mouse testis cDNA. The cDNA sequence of *Mouse Tudor Repeat-1 (Mtr-1)* was obtained by screening a fetal gonad cDNA library with this short EST. MTR-1 protein is present in the cytoplasm of spermatogonia, spermatocytes, and round spermatids and predominantly localizes to an intracellular structure known as the chromatoid body (Chuma *et al.* 2003)., Chromatoid bodies are electron-dense, amorphous material found close to mitochondria in spermatocytes and spermatids (Parvinen 2005). Small nuclear ribonucleoproteins (snRNPs) are other characteristic components of chromatoid body (Biggiogera *et al.* 1990). Results of immunostaining and co-immunoprecipitation using antibodies against MTR-1 and snRNPs revealed that MTR-1 and an assembled form of snRNPs co-localize in chromatoid bodies and form a complex. Additionally, when expressed in cultured cells, MTR-1 forms granules that co-localize with snRNPs in the cytoplasm during cell division. These results suggest that MTR-1 might function in assembling snRNPs into cytoplasmic granules in germ cells. Although *tudor* is essential for oogenesis in *Drosophila*, MTR-1 expression was undetecatable in mouse ovary by immunoblotting or immunostaining. It may be speculated that, if a null-mutant of *MTR-1* is generated in the mouse, it will have defects in spermatogenesis.

In summary, there is remarkable conservation between *Drosophila* and mammals of the genes expressed during germ cell development. But the functions of these genes diverge.

Thus, of the genes required for oogenesis in *Drosophila*, their mouse homologues appear to be essential for spermatogenesi but not necessary for oogenesis.

1.5.2 The vasa gene

Vasa is another gene first uncovered in *Drosophila*. It encodes an RNA helicase and is a component of the pole plasm that is inherited by and specifies the germ cells. VASA protein itself accumulates at the perinuclear nuage in the nurse cell and pole plasm at posterior oocyte, although the mRNA is uniformly distributed in the oocyte. *Vasa* is required for normal accumulation of several germ-line proteins and the function of pole plasm (Lasko & Ashburner 1988), and interaction with general translation factor eIF5B is necessary for VASA function (Johnstone & Lasko 2004).

Genetic homologues of *vasa* have been identified in many species, including *C. elegans*, *Xenopus*, zebrafish, mouse and rat, as well as humans (Roussell & Bennett 1993; Komiya *et al.* 1994; Yoon *et al.* 1997; Fujiwara *et al.* 1994; Castrillon *et al.* 2000). All of these *vasa* homologues have been found to be expressed specifically in germ cell lineages. Therefore, *vasa* is generally regarded as a germ line molecular marker. Drosophila *vasa* gene is mandatory for oogenesis, consistent with its role in mRNA localization. Knock-out mice presented a surprise, however. Female mice homozygous for the *Mvh (mouse vasa homologue)* mutation are fertile, whereas male mice homozygous for the *Mvh* mutation are sterile. Intriguingly, this divergence matches the pattern described above for other germ-line genes conserved between flies and mammals.

Northern and immunoblot analyses have revealed that *Mvh* mRNA and protein are expressed in mouse testis tissue (Fujiwara *et al.* 1994). *Mvh* mRNA is highly

concentrated within the spermatocytes prior to the first meiotic division, whereas relatively weaker signals are detectable in early round spermatids. No *Mvh* mRNA is detectable either in elongated spermatids or sperm or in the other cell types of the testis. MVH protein is present from the spermatocyte to round spermatid stages. In addition to a diffuse distribution of MVH protein in the cytoplasm, strong granular staining is observed in the perinuclear zone of the chromatoid body in round spermatids (Parvinen 2005). MVH antibodies have also been used to stain testicular sections of other mammals including rat, horse, pig, cow, monkey and human. Staining was detected in the cytoplasm of spermatogenic cells and notably in the chromatoid body in all species (Toyooka *et al.* 2000). Therefore, the expression pattern of VASA homologues during spermatogenesis is evolutionarily conserved in mammals.

Mvh expression has also been analyzed in the oocyte (Toyooka *et al.* 2000). MVH protein is present in the cytoplasm of growing oocytes, but not detectable in any ovarian somatic cell type. The strongest expression of MVH is detected in the cytoplasm of primordial oocytes, but its abundance decreases as the growth proceeds and it is undetectable in the cytoplasm of fully grown oocytes in antral follicle. Importantly, no particular cytoplasmic structure is found to be stained in oocytes at any stage, as examined by immuno-electron microscopy. This result may be contrasted to spermatogenesis where MVH is associated with the chromatoid body. This is consistent with the fact that no structure resembling a chromatoid body has been detected in mammalian oocytes. Moreover, germ cell determinants do not accumulate in the mammalian oocyte as they do in eggs of species such as *Drosophila*, *C. elegans* and *Xenopus laevis* where electron-dense accumulations, termed nuage, the French word meaning cloud (Massover 1968; al-Mukhtar&Webb

1971), that likely correspond to the chromatoid body are present (Tam & Zhou 1996a; Zernicka-Goetz 1998).

Targeted disruption of the *Mvh* gene results in serious defects in male germ cell development and in male sterility (Tanaka *et al.* 2000). The testis of homozygous null males is significantly decreased in size and produces no sperm. Histological examination has revealed that spermatogenesis stops before postmeiotic germ cells are produced (i.e., no spermatids or sperm). On postnatal day 15, part of the spermatocyte layer is lost in the homozygous null mutant testis. Analysis of the genes expressed in spermatocytes before pachytene stage shows there is no difference among genotypes. However, some genes expressed in pachytene stage spermatocytes are not detected in the mutant mice, implying that defects have occurred during this stage. Moreover, at 15 and 35 days after birth, TUNEL-labeled cells are found around 10-fold higher frequency in the homozygous testis as compared to the heterozygous testis, suggesting that the arrested germ cells undergo apoptotic cell death. In contrast to this dramatic phenotype on males, females lacking *Mvh* do not show any obvious reproductive defects and are fertile (Tanaka *et al.* 2000). Thus, *Mvh* is absolutely required for spermatogenesis but seems dispensable for oogenesis.

The human homologue of *vasa* is expressed in the germ cell lineage of both sexes (Castrillon *et al.* 2000). Human *vasa* mRNA is exclusively expressed in ovary and testis and can not be detected in somatic tissues. Results of immunostaining show that in the testis, VASA protein is expressed in spermatogonia, spermatocytes and spermatids. In spermatocytes, human VASA is most abundant in the chromatoid body (Toyooka *et al.* 2000). In the ovary, human VASA is expressed predominantly in the oocytes of

primordial follicles. In ovarian primordial follicles, VASA staining has been found in a compact perinuclear body that surrounds the oocyte nucleus. This body consists of a tight aggregate of membranes and vesicles with electron-dense material and has been termed the yolk nucleus. It is said to be present in oocytes of species ranging from spiders to humans and to be analogous to the sponge body in *Drosophila* (Hertig & Adams 1967; Wilsch-Brauninger *et al.* 1997).

In *Drosophila*, three classes of electron-dense particles have been identified: nuage particles, sponge bodies and polar granules. Nuage particles are VASA-positive and present around the perinuclear region of the nurse cells, polar granules are larger, VASA-positive, and localize at the posterior of the oocyte, and sponge bodies are EXU-positive and can be found throughout the cytoplasm of nurse cells. More recent work indicates that even though the morphology and localization of those RNPs are different, they might exchange positions and components frequently (Styhler *et al.* 2002). However, this early localization of VASA near the oocyte nucleus has not been reported in other species, and even in human, VASA immunostaining is homogenous throughout the cytoplasm in later stage oocytes(Castrillon *et al.*2000).

1.5.3 Nuage and chromatoid body

Nuage and chromtoid body are very important structure for gene regulation. In many animal species, there are distinctive electron-dense granules present in the cytoplasm of germ cells. These granules have been termed as nuage, the French word meaning cloud, because they have a cloudy appearance when observed under the microscope. There are several common features of nuage among different species. There is no regular shape and

no surrounding membranes, it is composed of RNA and proteins but not DNA, and it localizes near the mitochondrial cluster and nuclear envelope (Eddy 1975). Pole plasm in *Drosophila* is the best known form of nuage. In *Drosophila*, three proteins are known to localize to nuage: VASA, Aubergine and Tudor (Parvinen 2005). They are all components of polar granules and are mandatory for proper germ cell formation. The mutant phenotype of them suggests they function in post-transcriptional RNA processing.

Nuage has been identified in male germ cells of the mammal as a dense and fibrous material localizing in the perinuclear region. In these cells, it has been termed the chromatoid body. It was first described by Benda (Benda, 1891) and later by Niessing (Niessing, 1897) in spermatocyte of rat, mouse and several other mammals. It has not been detected in spermatogonia or in primary spermatocytes at early meiotic prophase (leptotene and zygotene stages) (Eddy 1974). However, it becomes prominent in early pachytene spermatocytes and remains in post-meiotic round spermatids. Its formation can first be recognized in early spermatocytes as several small particles associated with a mitochondrial cluster. In spermatids, it becomes discrete aggregated electron-dense perinuclear granules (Fawcett *et al.* 1970).

The chromatoid body is found to contain abundant RNA and ribonucleoprotein but no DNA (Paniagua *et al.* 1985; Biggiogera *et al.* 1990). Components of the chromatoid body so far identified include actin, cytochrome C, histone H4, transition protein-2 mRNA, RNA binding proteins p48/52, Sm proteins of snRNPs and core proteins of hnRNPs, ribosomal proteins P1/P2, 5S and 5.8S ribosomal RNAs, and MVH (Parvinen 2005). This suggests that the chromatoid body may function in storing mRNA that is to be used during the later stages of spermatogenesis.

One remarkable characteristic of the chromatoid body is its fast three-dimensional non-random movements in the cytoplasm of living cells. One possible engine of this movement is actin (Walt & Armbruster 1984). It has been found that there is a very close association of the chromatoid body with the nuclear envelope and Golgi complex (Thorne-Tjomsland *et al.* 1988). The rapid movements of the chromatoid body have been proposed to relate to the transportation of gene products in early spermatids (Parvinen *et al.* 1978). Under snap-freeze electron microscope, it was found that there was long contact region between the chromatoid body and the nuclear envelope. This observation may imply that there is interaction and transportation of material between the chromatoid body and the nucleus (Parvinen *et al.* 1997).

1.5.4 GUSTAVUS, a VASA-interacting protein

Recently, GUSTAVUS (GUS) was identified as a protein that interacts physically with VASA and is required for localization of VASA at the pole plasm of the fly and thus for specification of the germ cells (Styhler *et al.* 2002). GUS colocalizes with VASA in nuage particles around *Drosophila* nurse cell nuclei and transiently localizes to the pole plasm of the oocyte during oogenesis. *Gus* mutations block proper posterior localization of VASA, as does deletion of short region of GUS binding site of VASA. *Gus* mutants are female-sterile and heterozygotes produce embryos with fewer pole cells, as well as showing posterior patterning defects. Importantly, the mutant phenotype can be rescued by introduction of a *gus* transgene. Therefore, GUS is necessary for transportation of VASA and VASA localization to the posterior of oocyte depends on direct interaction with GUS in flies.

GUS contains two well-conserved protein domains: the SPRY domain, which was first identified in ryanodine receptors that mediate Ca^{2+} release from sarcoplasmic reticulum (Ponting *et al.* 1997) and now is thought to mediate protein-protein interactions (Wang *et al.* 2005), and a SOCS box, which has been implicated in ubiquitination of proteins, thus targeting them for proteasomal degradation (Kamura *et al.* 1998; Zhang *et al.* 1999). To determine whether a GUS homologue might be expressed and functional in germ cells in mice, we undertook a search for murine genes encoding proteins that were similar to Drosophila GUS. The research reported in this thesis identifies these genes as *SSB-1* and *SSB-4*. Therefore, in the following section, current knowledge of SSB-1 and SSB-4 is discussed.

1.6 The SSB family of proteins

The mouse and human genome each contains four proteins which have both a SPRY domain and a SOCS box. These comprise the SSB (SPRY domain and SOCS box containing protein) protein family and are termed SSB-1 through -4 (Masters *et al.* 2005; Wang *et al.* 2005). SSB-1 and SSB-4 both share around 70% sequence similarities with Drosophila *gus*. Protein conserved domains usually link to the protein function. Both SPRY domain and SOCS box have been identified in many mammlian proteins and SPRY domain was found to be a general protein-protein interface and many SOCS box proteins funtion as negative regulators of cytokine signalling.

1.6.1 The SPRY domain

The SPRY domain was first identified in a *Dictyostelium discoideum* kinase, splA, and in mammalian Ca^{2+}-release channels ryanodine receptors (RyR). The SPRY domain is ~110 amino acids and is present in three copies in each of the three mammalian RyR subtypes. The triplicated domain is located in the central region of the RyRs and is also present three times in splA (Ponting *et al.* 1997). There are 149 human proteins so far known to contain the SPRY domain (Woo *et al.* 2006).

The SPRY domain has recently been predicted to act as a general protein-protein interface. For example, the SPRY domain-containing proteins, RanBP9 and RanBP10, specifically bind the tyrosine kinase domain of MET via their SPRY domain (Wang *et al.* 2002; Wang *et al.* 2004). MET is a receptor tyrosine kinase (RTK) for hepatocyte growth factor (HGF). The MET receptor contains an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. MET receptor dimerizes under the induction of HGF and dimerized MET proteins result in the

activation of tyrosine kinase domain then of a signaling cascade, including the Ras pathway which is implicated in many human tumors (Stella & Comoglio 1999).

RanBP9 was initially found to interact with MET protein by the yeast two-hybrid system (Wang *et al.* 2006). The interaction between MET and RanBP9 was then confirmed by *in vitro* GST-pull down assay and co-immunoprecipitation. That binding of RanBP9 to MET was due to the SPRY domain was proved by constructing a truncated form of RanBP9 and showing that RanBP9 without the SPRY domain could not interact with MET in the two-hybrid system. Furthermore, the GST-SPRY domain itself can pull down MET. Using constructs encoding truncated forms of MET in GST pull down assays, the tyrosine kinase domain of MET was identified as the region that interacts with the SPRY domain (Wang *et al.* 2002).

All four SSB proteins interact with the MET tyrosine kinase, as established using GST pull-down and co-immunopreciptation assays (Wang *et al.* 2005). Additionally, experiments using truncated proteins established the SPRY domain as required for the interaction. Importantly, SSB-1 also participates in the regulation of MET signalling. The interaction of SSB-1 and MET is positively regulated by HGF. For example, HGF-induced ERK phosphorylation in HEK293 cells is enhanced 5-fold when SSB-1 is overexpressed in the cells. Conversely, suppression of SSB-1 by RNA interference down regulates HGF-induced serum-response-element (SRE)-luciferase activity and decreases Elk-1 activation. These results suggests that SSB-1 enhances the activity of the HGF-MET induced Erk-Elk-1-SRE pathway (Wang *et al.* 2005).

A very recent publication has shown that Drosophila VASA and GUS also interact through SPRY domains (Woo *et al.* 2006). Combined biochemical and biophysical methods were used to demonstrate the structure, the domain boundaries, and the target protein-binding surface of the SPRY domain of GUS. Therefore, we believe there will be more evidence to prove that SPRY domain as an interface for protein interaction in the future.

1.6.2 The SOCS box

The SOCS box is a motif of ~50 amino acids in length. The C-terminal half is rich in leucine and proline residues (though its function is unknown), while the N-terminal half region comprises a consensus BC element, so named because it is found in elongin B and elongin C, two important proteins in E3 ubiquitin ligase complexes (Zhang *et al.* 1999). About forty SOCS-box proteins have so far been identified in humans (Kile *et al.* 2002). Based on their other domain structures, the SOCS box protein family has been divided into nine structural classes (Figure 6). These are defined as the traditional SOCS box protein family, which contains an SH2 domain; the ASB family, which contains an ankyrin repeat; the WSB family, which contains a WD40 repeat; the SSB family that contain a SPRY domain; Ras and some ras-like GTPases that also contains a SOCS box; the tubby-domain-containing proteins with a SOCS box; the neutralized family of proteins with SOCS box; the von-Hippel-Lindau (VHL) tumor suppressor protein with SOCS box; and several miscellaneous proteins with SOCS box including MUF-1 and elongin A. In almost all of these proteins, the SOCS box is located in the C-terminal (it is

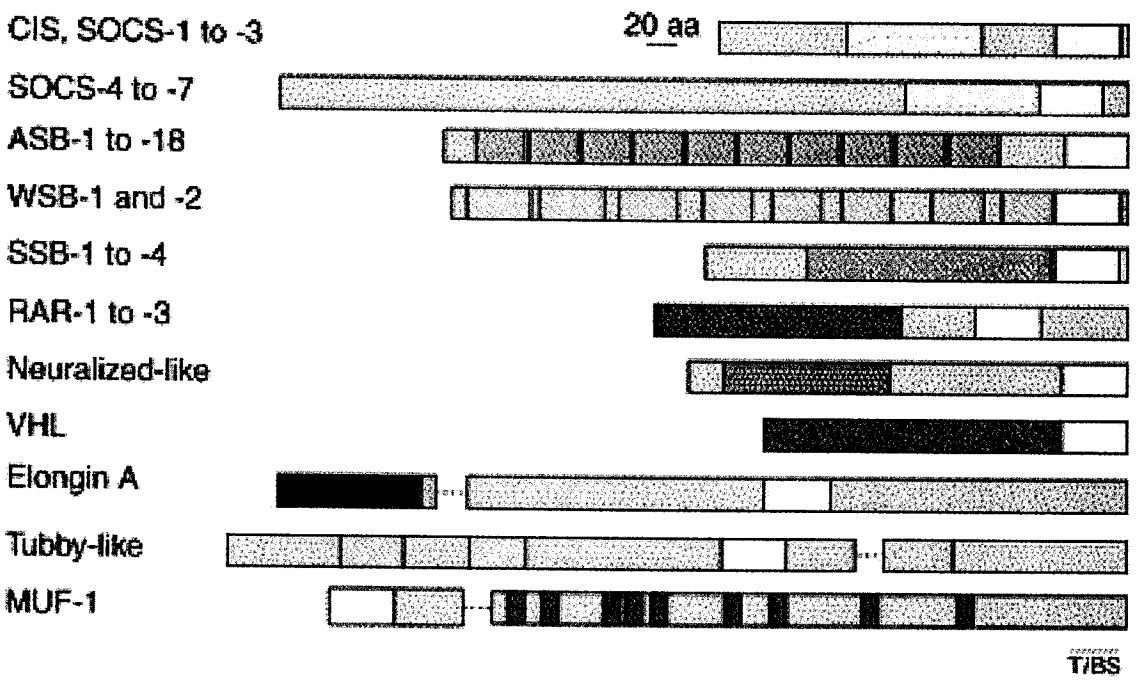


Figure 6: The domain structure of SOCS-box-containing proteins.

The yellow box represents the SOCS-box motif. The Src-homology 2 (SH2) domains are shown in lime green. The ankyrin repeats in ASB family are shown in grass green. The WD40 repeats in WSB family are shown in teal. The SPRY domains in SSB family are shown in lilac. The GTPase domain in RAR is shown in purple. The Neuralized domain of the Neuralized-like protein is shown in pink. The β-domain of VHL tumor suppressor protein is shown in cerise. The elongation factor SII homology domain in elongin A is shown in brown. The Tubby domain is shown in camel. The leucine-rich repeats in MUF-1 are shown in charcoal. Taken from Kile et al. 2002.

at the N-terminal in MUF-1 and in the middle in ElonginA and Tubby-domain containing protein).

Some SOCS proteins play a clear role in negative regulation of cytokine signalling. CIS (cytokine inducible SH2-containing protein) was the first SOCS protein identified as a negative regulator of cytokine signaling (Yoshimura *et al.* 1995). Genetic deletion of *Socs1* results in neonatal death caused by excessive gamma interferon (IFN-R) signalling (Starr *et al.* 1998). *Socs3* plays a central role in inhibiting interleukin-6 and granulocyte colony-stimulating factor signaling (Roberts *et al.* 2001).

SOCS box proteins of WSB, ASB and ras family were found to interact with the ElonginBC complex and to function as adaptors in proteasomal degradation pathways (Zhang *et al.* 1999; Kamura *et al.* 1998). It is predicted that the C-terminal of SOCS box recruits E3 proteins, whereas the N-terminal domains may interact with the targeting protein to be degraded (Nicholson *et al.* 2000). Thus, these SOCS proteins regulate protein turnover by targeting proteins for polyubiquitination and proteasome-mediated degradation. However, the functions of most family members, including SPRY domain SOCS box protein family discussed in this thesis, remain to be determined.

1.6.3 The first physiological analysis of mammalian SSB function

There are four SSB protein family members in the mouse, as in the human. Sequence alignment shows that in mice, SSB-1 and SSB-4 share 75% sequence similarity and SSB-2 and SSB-3 share 42% similarities. Quantitative analysis of mRNA levels show that *SSB-1*, *SSB-2* and *SSB-3* are expressed in several different mouse tissues. In contrast,

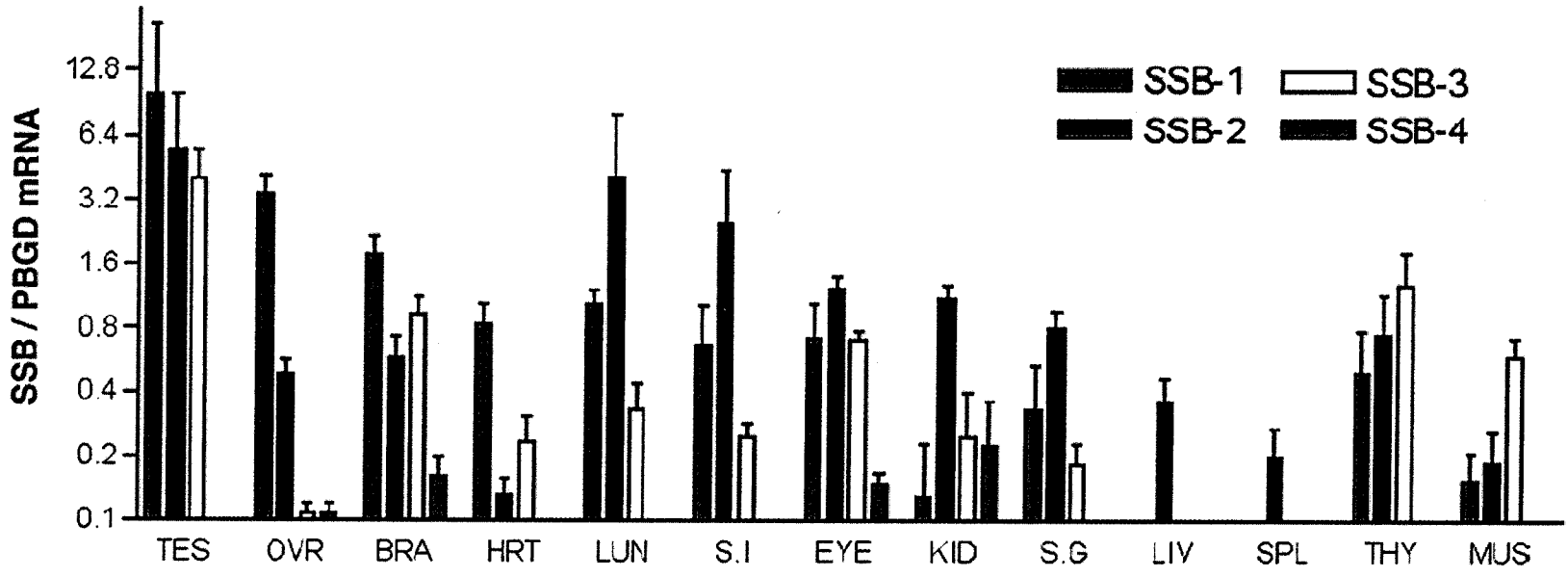


Figure 7: The expression of SSB mRNA in adult mouse tissues.

SSB mRNA levels were quantitated by real time RT-PCR (normalized against PBGD mRNA levels). Abbreviations were used for testes (TES), ovary (OVR), brain (BRA), heart (HRT), lung (LUN), small intestine (S.I), kidney (KID), salivary gland (S.G), liver (LIV), spleen (SPL), thymus (THY), and muscle (MUS). At least three mice were used for each sample. *SSB-1*, *SSB-2* and *SSB-3* are all expressed in several mouse tissues, whereas expression of *SSB-4* is barely detectable in most tissues. *SSB-1*, *SSB-2* and *SSB-3* are all highly expressed in testis at similar levels, which might indicate functional redundancy in this tissue. *SSB-1* was also very abundant in the ovary and heart. Taken from Masters *et al.*2005.

expression of *SSB-4* is usually low. *SSB-1*, *SSB-2* and *SSB-3* are all highly expressed in testis at similar levels. *SSB-1* was most abundant in the ovary and heart, *SSB-2* was mainly in the lung, kidney, liver, and spleen, and *SSB*-3 was predominant in muscle (Figure 7). Several SSB proteins expressed in the same cell type might indicate they are functionally redundant in that cell type, whereas specific expression of a single SSB protein in a particular tissue or cell might imply that it has a specific function in that cell (Masters *et al.* 2005).

Creating the *Ssb-2* gene knockout mice represents the first physiological analysis of mammalian SSB function. The gene was deleted by homologous recombination (Masters *et al.* 2005). The construct was designed to delete the entire region coding for SSB-2 and was transfected into C57BL/6 ES cells. Targeted ES cells were then injected into blastocysts to generate chimeric mice and homozygous mutant mice were obtained by mating F_1 heterozygous for the *Ssb-2* deletion. Both male and female mice homozygous for the deletion of *Ssb-2* were fertile. To probe the probable distribution of SSB-2, a reporter gene (*ß*-galactosidase) was placed under control of the endogenous *Ssb-2* promoter. Histological examination showed that all organs and tissue types expressed ß-galactosidase and are correlated with results of quantitative RT-PCR(Masters *et al.* 2005).

Because significant expression of SSB-2 was observed in megakaryocytes, analysis of platelets and megakaryocytes in SSB-2-knockout mice was performed. These were found to have a reduced rate of platelet production (25% decrease in circulating platelets) compared to wild type and heterozygous mice, resulting in very mild thrombocytopenia. Most other peripheral blood parameters were unaltered and the morphology of platelets appeared normal by electron microscopy. However, the rate of platelet production was

lower than normal, indicating that fewer platelets per megakaryocyte were produced. In addition, blood urea nitrogen (BUN) levels were decreased by 23% in homozygous mice lacking SSB-2 compared to wild type mice. Therefore, SSB-2 plays a central role in regulating platelet production and BUN levels. SSB-2 might function through different signalling pathways because no known biochemical pathway links thrombocytopenia and BUN decrease. Identifying new SSB-2 interacting proteins and clarifying the relevant signalling pathway will help us understand the function of SSB-2 more clearly and might give us some clues regarding the physiological significance of other mammalian SSB protein family members.

1.7 Objective of the thesis

Many genes play conserved roles in different species. As many genes involved in fly oogenesis have homologues in mammals, it may be proposed that these homologues may also play key roles in mouse gametogenesis. Several murine homologues of Drosophila genes that play important functions in germ line development have been discussed. GUS plays an important role in oogenesis in the fly. The aim of the research described here was to identify GUS homologues and their function in mice. These homologues were identified as members of the SSB protein family, whose function is poorly understood. The results set the stage for analyzing the function of SSB proteins during germ cell development in mammalian males and females.

2. MANUSCRIPT I

Abstract

Mammalian homologues of genes that control oogenesis in other organisms may play similar roles in mammalian ovarian development. In *Drosophila*, GUSTAVUS (GUS) protein physically interacts with and is necessary for the proper posterior localization of VASA protein, and thus is required for specification of germ cells. We identified two mouse genes, *SSB-1* and *SSB-4* (SPRY domain SOCS box protein), whose protein products share 75% identity and are each approximately 70% identical to *Drosophila* GUS. Both SSB-1 and SSB-4 mRNA were detectable in mouse ovaries by Northern blotting of total and poly(A)+ RNA, but were expressed in few other tissues. SSB-1 was detectable in testes, although the 3'-untranslated region of the mRNA was considerably shorter than the ovarian mRNA. *In situ* hybridization and RT-PCR analysis of ovaries revealed that both genes were expressed in granulosa cells at all stages of follicular development. In contrast, expression was barely detectable in oocytes. Immunoblotting analysis revealed that SSB-1 protein was present in follicles at different stages of growth, and immunocytochemistry confirmed that SSB-1 and SSB-4 were detectable in granulosa cells of primary and subsequent stage follicles and that they were present in both mural and cumulus granulosa cells of antral follicles. These results establish that GUS-related proteins, which in Drosophila are restricted to the germ cells, are in the mouse instead expressed in the granulosa cells and are present throughout folliculogenesis. Based on their tissue-restricted pattern of expression and apparent abundance in granulosa cells, we propose that SSB-1 and SSB-4 play key roles in regulating granulosa cell physiology.

Introduction

Development of a mature fertilizable oocyte requires the coordinated expression and interaction of a variety of gene products in the oocyte and the surrounding granulosa cells. Understanding the molecular basis of follicular development is a prerequisite for revealing the genetic basis of differential fertility, the etiology of some types of infertility, and for improving assisted reproductive technology. To this end, several strategies have been developed to identify oocyte-specific genes whose function can then be experimentally addressed. One approach has used *in silico* analysis of public databases to identify expressed sequence tags that are enriched in cDNA libraries from oocytes (Rajkovic *et al.* 2001): this has uncovered a number of key genes required for oocyte growth (Rajkovic *et al.* 2004). A second approach has been to use subtractive hybridization procedures to identify transcripts enriched in oocytes compared to another cell type: this has also yielded previously unknown oocyte-specific genes (Vallee *et al.* 2005; Zeng & Schultz 2003).

Another strategy is to search for mammalian homologues of genes already known to play key roles during oogenesis in other organisms. The potential advantage of this approach is that the gene candidates need not be restricted to those expressed mainly or exclusively in the oocyte. Moreover, they can be selected from genes that have already been demonstrated experimentally to play a role in oogenesis. A rich source of these genes is the fly, *Drosophila melanogaster*, in which the genetic basis of oogenesis has been intensively studied. For example, an apparent mammalian homologue of the *Drosophila nanos* gene, which is required in the fly to prevent germ-line stem cells from undergoing

differentiation (Wang & Lin 2004), is required in mice for the proliferation or migration of primordial germ cells (Tsuda *et al.* 2003). Genes closely related to *Drosophila tudor*, which encodes a component of the pole plasm where the primordial germ cells develop, and *staufen* have also been identified in mammalian male germ cells, although their function in mammals is not yet known (Chuma *et al.* 2003; Saunders *et al.* 2000; Smith *et al.* 2004).

vasa is another gene first uncovered in *Drosophila*. It encodes an RNA helicase and is a component of the pole plasm that is inherited by and specifies the germ cells. *vasa* is required to establish localized translation of at least two mRNAs, *oskar* (no known mammalian homologue) and *nanos*, within the polar plasm. VASA protein itself accumulates at the pole plasm, although the mRNA is uniformly distributed in the oocyte (Johnstone & Lasko 2001). A murine homologue of *vasa* (mouse vasa homologue, *Mvh*) has been identified (Fujiwara *et al.* 1994). *Mvh* is expressed in embryonic germ cells as well as in small oocytes and in male germ cells. Genetic deletion of *Mvh* caused arrest of developing spermatocytes around the stage of pachytene but, surprisingly, has no apparent effect on oogenesis (Tanaka *et al.* 2000).

Recently, a novel protein in the fly, termed GUSTAVUS (GUS), was identified that interacts physically with VASA and is required for localization of VASA at the pole plasm and thus for specification of the germ cells (Styhler *et al.* 2002). GUS contains two well-conserved protein domains – a SPRY domain, which was first identified in ryanodine receptors and is thought to mediate protein-protein interactions (Wang *et al.*

2005), and a SOCS box, which has been implicated in ubiquitination of proteins, thus targeting them for proteasomal degradation. To determine whether a GUS homologue might be expressed and functional in germ cells, we undertook a search for murine genes encoding proteins that were similar to GUS. We report that the protein products of the genes, *SSB-1* and *SSB-4*, bear substantial similarity to GUS. Unexpectedly, although *SSB-1* and *SSB-4* are expressed in the ovary, they are barely detectable in the germ cells. Rather, and in contrast to *gus*, they are expressed in granulosa cells of the ovarian follicle. We discuss potential roles of these proteins in granulosa cell function.

Materials and Methods

Animals

Experiments were carried out using CD-1 mice (Charles River, St-Constant, QC). All animal procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the McGill University Health Centre.

Northern blot hybridization

Sequences within the coding sequences of *SSB-1* and the coding and part of the 3'-untranslated region of *SSB-4* were amplified by the polymerase chain reaction (PCR). Antisense RNA probes were prepared from the PCR products by ligation to a T7 promoter adapter (Lig'nScribe, Roche Applied Science, Montreal, QC, Canada) followed by incubation with T7 RNA polymerase (Roche) in the presence of digoxygenin-labelled UTP (Roche). Mouse total RNA from different tissues (Ambion, Austin, TX, USA) was stored at –80°C. RNA (2 μg/lane) was separated on denaturing agarose gels, transferred by downward capillary blotting (Turboblotter, Mandel Scientific, Guelph, ON, Canada) to a nylon membrane (Roche) and fixed by exposure to ultraviolet light. Membranes were hybridized with the RNA probes and bound probe visualized using a commercial detection kit (Roche). Poly(A)+ RNA was isolated from total RNA of mouse liver, ovary and kidney (Oligotex, Qiagen) and analyzed as for total RNA.

In situ hybridization

Antisense and sense RNA probes were prepared as described above. Ovaries were fixed in freshly prepared 4% *para*-formaldehyde, dehydrated, embedded in paraffin, sectioned

at 7 μm, and mounted on glass slides. Following rehydration, hybridization was carried out overnight at 45°C in hybridization buffer (40% deionized formamide, 10% dextran sulfate, 4X SSC, 1X Denhardt's solution, 10mM DTT, 1 mg/ml yeast DNA, 1 mg/ml denatured and sheared salmon sperm DNA). Probe was present at 0.5-1 ng/μl hybridization buffer. Bound probe was revealed using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). Activity was detected using NBT/BCIP (Roche) as the chromogen following the manufacturer's directions.

Collection of ovarian oocytes and ovulated eggs

Granulosa-oocyte complexes were collected from mice up to 3 weeks of age by either enzymatic digestion of the ovaries or by puncturing the follicles with a 30G needle, as previously described (McLay & Clarke 1997). To collect ovulated eggs at metaphase II, 7- to 8-week old female mice were injected with 7.5 IU of pregnant mares' serum gonadotropin (Sigma Chemicals, Windsor, ON, Canada) followed by 5 IU of human chorionic gonadotropin (hCG) 44 hr later to induce superovulation. Egg masses were recovered at 16 hr post-hCG and oocytes were freed from the granulosa cells using hyaluronidase. The granulosa-oocyte complexes or oocytes were transferred using a pipette to storage at –80° for up to four weeks.

Reverse transcription and polymerase chain reaction

RNA was extracted from oocytes using Trizol (Invitrogen, Burlington, ON, Canada) and reverse-transcribed into cDNA using MMLV as previously described (Mohamed *et al.*, 2004). cDNA corresponding to 15 oocyte-equivalents was subjected to PCR using a

thermal cycler (Biometra UNO Thermoblock, version 3.30) using the following conditions: 95°C for 5 min and (94°C for 45 sec, 56°C for 45 sec, 72°C for 1 min) x 35 cycles; the last cycle was followed by a 5-min extension at 72°C.

Generation of antibodies against SSB-1 and SSB-4

Peptides corresponding to amino acids 15-31 (DPTYRPLKQELQGLDYC) of SSB-1 and 16-32 (EPALRPAKRELRGLEPG) of SSB-4 were synthesized and injected into rabbits to generate antibodies (Biosynthesis, TX, USA). Affinity-purified antibodies were prepared by the supplier.

Immunoblotting and immunoprecipitation

Ovaries were rapidly homogenized in 2X loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.1% bromophenol blue, 10% β-mercaptoethanol), denatured at 95°C for 5 minutes, and centrifuged to pellet debris. Granulosa cell, granulosa-oocyte, and oocyte extracts were prepared by transferring the cells into an Eppendorf tube, withdrawing excess medium, and adding an appropriate volume of sample loading buffer, and denaturing as above. Protein electrophoresis, transfer to PVDF membranes and immunoblotting were carried out as previously described (Allard *et al.* 2002). Primary antibodies were used at a dilution of 1:1000, secondary antibodies conjugated to horseradish peroxidase (HRP, Promega, Montreal, QC, Canada) at a concentration of 1:5000. HRP activity was revealed using ECL+ (Amersham, Montreal, QC, Canada) following the manufacturer's directions.

For immunoprecipitation, ovaries of 8-week old CD-1 mice were homogenized in cold lysis buffer (50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 1% NP-40) with one tablet of protease inhibitor cocktail (Roche) added to every 10 mL buffer. The sample was then centrifuged at 14,000g at 4°C for 15 minutes, and the supernatant was collected. Ten μg of antibody was added to the supernatant (containing 5-10 mg total protein) in a final volume of 600 μl and mixed by rotation for 4 hr at 4°C. 150 μl of Protein A-Sepharose beads (Upstate, Charlottesville, VA, USA) were washed 4 times with PBS, then twice with lysis buffer, and added to 150 μl of the protein-antibody mixture. This was mixed by rotation overnight at 4°C. Following a brief centrifugation, the supernatant was removed and the beads washed 3 times with lysis buffer. 150 μl of 2X loading buffer was added to the beads and the mixture was boiled for 5 minutes. Samples were run on 10% SDS-polyacrylamide gels and subjected to immunoblotting.

Immunostaining

Ovaries were excised and fixed overnight at 4°C in freshly prepared 4% *para*-formaldehyde, washed in PBS, dehydrated, and embedded in paraffin. Five micron sections were exposed to 3% H_2O_2 in absolute methanol for 10 minutes, then washed 3 times in H_2O. The slides were placed in an antigen retrieval solution (9 ml 0.1M citric acid and 41 ml 0.1M sodium citrate in 450 ml H_2O) and boiled for 10 min. After cooling, the slides were washed 3 times in PBS containing 0.1% Tween-20 (PBST). They were treated with a blocking solution (PBST, 3% bovine serum albumen) for 1 h, then incubated overnight at 4°C with affinity-purified anti-SSB diluted 1:200 in blocking solution. After three 30 min washes in PBST, the sections were incubated with biotinylated secondary antibodies (ABC kit, Vector Laboratories), washed, and incubated with the ABC complex following the manufacturer's instructions. The sections were

washed and stained using the AEC color substrate (Vector Laboratories, Burlington, ON, Canada), then washed and counterstained with hematoxylin for 30 sec. Slides were mounted in glycerol.

Cell culture and transfection conditions

KK-1 and NT-1 cells were generously supplied by Prof. I. Huhtaniemi (Imperial College London, UK). HeLa and DC3 cells were generously supplied by Prof. R. Farookhi (McGill University). CHO cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM, or DMEM/F12 for the KK-1 and NT-1 cells, supplemented with 10% fetal bovine serum and transfected at 70-90% confluency. The coding sequences of *SSB-1* and *SSB-4* were inserted into pTriEX-2 and sequenced to confirm their identity. For transfection, 4 μg of plasmid and 10 μl of lipofectamine (Invitrogen) were incubated separately in serum-free medium for 5 minutes, combined and allowed to stand for 20 minutes, then added drop-wise to the plate containing the cells. Culture medium was changed 6 hr after transfection. Cells were harvested by scraping or trypsinization 24 hr and 48 hr after transfection.

Primers

F: Forward primers; R: Reverse primers. The primers for Northern blot were used to PCR-amplify the probe for hybridization.

Northern analysis:

SSB-1 (497 nt)

F: ATAACGACCGTTCGCTCAAC; R: AGTCCGTTCAGTAGCGCAT

SSB-4 coding region (823 nt)

F: CGGGATCCATGGGTCAGAAGC; R: CCCAAGCTTTCACTGGTACTG

SSB-4 3'-UTR (533 nt)

F: AAAAGCACCTGGCCTTACCT; R: CACAAAGATGCCAAATGGTG

In situ hybridization:

SSB-1 (236 nt)

F: GGCGTAACCGTCTCTACCAC; R: AGTCCGTTCAAGTAGCGCAT

SSB-4 coding region (280 nt)

F: GCACCCAGTAGCCCAGAGCA; R: AAGGAATCTGGCAGAGCAAA

SSB-4 3-UTR (289 nt)

F: AAAAGCACCTGGCCTTACCT; R: CTTCACCCTGAATGATGGCT

RT-PCR (each pair spans an intron):

ZP3 (467 nt)

F: GCACCTTCCTACTCCACGAC; R: ATCCACCGTGAACTGGAGAG

FSH-R (401 nt)

F: GAGGCCTTCCAGAATCTTCC; R: CTGGCCCTCAACTTCTTCAG

SSB-1 (558 nt)

F: ATAACGACCGTTCGCTCAAC; R: GAACGCCGGCACAGGTCCAT

SSB-4 (576 nt)

F: CCGGATACAAGTCGAGAGGA; R: TGCTCTAGGCTACTGGGTGC

Results

Homologues of the *Drosophila gus* gene are present in the mouse and other vertebrates

To search for mouse genes related to *gus*, we screened GenBank using the full-length Drosophila *gus* sequence (CG2944_RB). This screen identified two closely related genes, *SSB-1* (SPRY-domain SOCS box protein 1, NP_083311) and *SSB-4* (NP_660116), whose protein products are approximately 75% identical and each approximately 70% identical to Drosophila GUS (Figure 1A). Further screening using the SSB-1 sequence revealed apparent SSB-1 homologues in both mammalian and non-mammalian species (Figure 1B). All of the SSB-1-related proteins contain a SPRY domain and a SOCS box, as shown in the Figure. Moreover, the protein sequences are remarkably conserved. Mouse SSB-1, for example, is 98% identical to human SSB-1 and 89% identical to *Xenopus* SSB-1. Thus, SSB-1 and presumably SSB-4 are to be highly conserved. Two other SSB family proteins, SSB-2 and SSB-3, that share less sequence similarity with SSB-1 and SSB-4 have also been identified (Wang *et al.* 2005).

SSB-1 and *SSB-4* mRNAs are expressed in the mouse ovary and testis

To examine the expression of *SSB-1* and *SSB-4* in mouse tissues, we screened Northern blots using probes corresponding to the coding regions of *SSB-1* and *SSB-4* and the 3'-untranslated region (UTR) of *SSB-4*. Among the ten tissues screened, the *SSB-1* probe detected a single band of about 3 kb in heart and ovary and of about 1.4 kb in testis (Figure 2). No expression was detectable under these conditions in liver, brain, thymus,

lung, spleen, or kidney. To confirm that the RNA detected was potentially translatable, poly(A)+ RNA from the mouse liver, ovary and kidney was isolated and probed. *SSB-1* transcripts were detected as a prominent 3 kb band in ovary but not in liver and kidney (Figure 2).

By comparing the published cDNA (BC057563) and genomic (NT_039258.4) sequences of *SSB-1*, we were able to map a putative exon-intron structure (Figure 3A). To determine which exons were represented in the *SSB-1* expressed in ovary and testis, we designed primers within the different putative exons and used these to PCR-amplify cDNA from these tissues (data not shown). The results revealed that *SSB-1* expressed in the ovary consists of either exon 1 or exon 2 spliced to exon 3 and exon 4 (Figure 3B). Transcripts containing both exon 1 and exon 2 were not detected. *SSB-1* expressed in the testis comprised exons 2 and 3 together with a truncated fragment of exon 4. This truncation appears to arise from the use of a polyadenylation signal that is not used in the ovarian transcript. The absence of most of exon 4 from the testicular transcript accounts for its relatively small size. These differences in primary sequence of the transcripts exist solely within the 5'- and 3'-untranslated regions; the coding sequence of all transcripts is identical.

Using the *SSB-4* coding-region probe, a single 2.8 kb band was detected in ovary and also in brain, heart, kidney, and embryo (Figure 2). Two transcripts (2.8 kb and 4.7 kb) were detected in thymus. *SSB-4* was not detected in liver, lung, spleen, or testis (Figure 2). Analysis of poly(A)+ mRNA confirmed that a 2.8 kb *SSB-4* transcript was present in

ovary and kidney, but not in liver (Figure 2). As *SSB-4* expression was relatively weak when total RNA was analyzed, these analyses were repeated using a probe from the 3'-UTR of *SSB-4*. The same expression patterns were observed (data not shown). These results indicate that *SSB-1* is expressed in mouse gonadal tissue of males and females, and *SSB-4* in the ovary, as well as in a small number of other tissues.

SSB-1 and *SSB-4* mRNAs are detectable in ovarian granulosa cells but not oocytes

To define the cellular localization of *SSB-1* and *SSB-4* transcripts in mouse ovary, *in situ* hybridization using digoxigenin-labeled RNA probes was performed. Hybridization signals corresponding to *SSB-1* were highly concentrated in the granulosa cells of follicles at different stages of growth (Figure 4A), whereas no signals above background were detected in oocytes or the stromal cells. A similar pattern of expression was observed for *SSB-4* (Figure 4B). This result was unexpected in view of the results obtained in *Drosophila*, where *gus* expression is also in the developing oocyte (Styhler *et al.*, 2002). Therefore, to clarify the sites of *SSB-1* and *SSB-4* expression in the mouse, fully grown oocytes and their surrounding granulosa cells were collected and analyzed separately using RT-PCR (Figure 4C). PCR products corresponding to *SSB-1* and *SSB-4* were observed in granulosa cells, as was a product corresponding to the FSH receptor that is known to be expressed in these cells. Expression of the zona pellucida-3 (*zp-3*) gene was not detected, confirming that the granulosa cells were not contaminated with oocytes. In contrast, neither *SSB-1* nor *SSB-4* transcripts were detected at a significant level in oocytes, whereas *zp-3* expression was detected. Nonetheless, in the case of SSB-4, the

RT-PCR analysis did reveal a weak signal in oocytes (Figure 4C). These results indicate that the granulosa cells are the major site of expression of *SSB-1* and *SSB-4* in the ovary.

SSB-1 and SSB-4 proteins are detectable in granulosa cells but not oocytes

During development of the follicle to the antral stage, the granulosa cells become separated into two morphologically distinct sub-populations – the cumulus granulosa that surround the oocyte and the mural granulosa that lie inside of the basement membrane-thecal cell layer that constitute the follicular wall. The mural cells are sometimes further subdivided according to whether they contact the antrum or the follicle wall. Molecular and functional differences exist between the cumulus and mural granulosa (Eppig *et al.* 2005; Joyce *et al.* 2001). To investigate whether SSB-1 protein was expressed throughout the follicle, a polyclonal antibody was raised against a short peptide corresponding to its N-terminal region that is relatively dissimilar to SSB-4 (amino acids 15-31, see Figure 1) and affinity-purified it.

Anti-SSB-1 antibodies recognized a protein migrating at about 31 kDa in CHO cells harvested 24 hr after transfection with a construct encoding SSB-1, migrating at just above 40kDa in CHO cells transfected with a construct encoding SSB-1 with a 100 amino acid tag but not in non-transfected cells (Figure 5A). Forty-eight hr after transfection, a 36 kDa protein had appeared. These results imply that SSB-1 can be modified intracellularly to generate a slower-migrating form. This antibody also recognized a species of about 40 kDa in non-transfected CHO cells and in tissues where we did not detect SSB-1 mRNA (data not shown). Similarly, transfected HeLa cells also displayed several slow-migrating

species in addition to the predicted 31 kDa protein (Figure 5B). Taken together, these results indicate that SSB-1 can be modified within the cell to generate multiple forms that differ in M_r.

When whole-ovary extracts were probed using anti-SSB-1, both the 31 kDa and 36 kDa bands were detected, as well as the apparently unrelated 40 kDa band (Figure 6A). To verify the ovarian cell type expressing SSB-1, granulosa cell-oocyte complexes were collected and either used directly for blotting or separated into oocytes and granulosa cells and analyzed separately. As shown in Figure 6B, the 31 kDa and 36 kDa species were present in the granulosa-oocyte complexes and the purified granulosa cells but not in the purified oocytes. To confirm these results, we also analyzed SSB-1 expression in immortalized cell lines derived from granulosa cells (Havelock *et al.* 2004). KK-1 and NT-1 cells were derived from granulosa cell tumours of transgenic mice expressing SV40 T-antigen under the control of the inhibin α-subunit promoter (Kananen *et al.* 1995; Rilianawati *et al.* 1999). DC3 cells were derived from rat granulosa cells transformed using SV40 in vitro (Fitz *et al.* 1989). All three lines expressed the 31-kDa species as well as species migrating near 36 kDa. The slight differences in electrophoretic mobility of the 36-kDa species observed in the different lines may reflect post-translational modification. Taken together, these results are consistent with the data obtained by *in situ* hybridization analysis and RT-PCR, and reinforce the conclusion that SSB-1 is expressed in granulosa cells of growing follicles but not in female germ cells.

To determine whether expression of SSB-1 was developmentally regulated during follicular growth, we took advantage of the fact that, during the first three weeks of life, a relatively large pool of follicles undergoes synchronous growth. Thus, ovaries obtained from mice of increasing age during this time are enriched for follicles at successively more advanced stages of development. We isolated granulosa-oocyte complexes from 5- to 20-day old mice. SSB-1 was present in complexes at all stages of growth (Figure 6D). In addition, we stained ovarian histological sections using anti-SSB1. Strong staining was detected in granulosa cells of growing follicles and in both the mural and cumulus granulosa of antral follicles (Figure 7A, B), but not when the primary antibody was replaced by rabbit immunoglobulin at the same concentration (Figure 7D). Moreover, the same staining pattern was observed using a different antibody raised against the N–terminal 60 amino acids of SSB-1 (data not shown). Similarly, antibodies raised against SSB-4 that recognized transfected SSB-4 but not SSB-1, confirming their subtype specificity (data not shown), stained the granulosa cells of growing and fully grown follicles (Figure 7C). Figure 7 also shows a weak staining reaction in the oocytes. This staining was also observed when the primary antibody was omitted (see Figure 7D), indicating that the detection system produces a certain degree of non-specific staining in the oocyte cytoplasm. Taken together, these results indicate that SSB-1 and SSB-4 are expressed in granulosa cells in primary follicles and all subsequent stages and in both the mural and antral granulosa of antral follicles.

Discussion

We have identified two members of the *SSB* gene family that are very similar in sequence and presumably homologous to the *gus* gene of *Drosophila*. All of these proteins carry a SPRY domain and a SOCS box. Sequence similarity is strongest in the SPRY domain and is significant in the SOCS box. Moreover, SSB-1 is highly conserved among mice, humans, and *Xenopus*. In addition, SSB-1 and SSB-4 proteins are about 70% identical to *Drosophila* GUS. This is considerably higher than the protein sequence identity between MVH, NANOS, and TUDOR and their apparent homologues in *Drosophila*. Taken together, these results suggest that the biochemical function of GUS is conserved in SSB-1 and SSB-4 and that this function is likely important in a variety of organisms.

By Northern analysis, we observed that *SSB-1* and *SSB-4* were expressed in specific tissues. This result differs slightly from a recent database analysis indicating that these genes are expressed in many tissues (Wang *et al.* 2005). This suggests that, notwithstanding a widespread low-level expression of these genes, the major sites of *SSB-1* and *SSB-4* function may be restricted to a few tissues. One of these tissues is likely to be the ovary, where both *SSB-1* and *SSB-4* mRNA and protein were abundantly expressed in the granulosa cells of growing follicles. Moreover, expression was detected in granulosa cells from follicles of different sizes, suggesting that *SSB-1* and *SSB-4* are constitutively expressed in these cells, at least from the earliest stages of follicle growth.

Expression of *SSB-1* and *SSB-4* in ovarian granulosa cells was unexpected, as it contrasts with the expression of *gus* in Drosophila, which is restricted to the oocyte and nurse cells (Styhler *et al.* 2002). We could not detect *SSB-1* mRNA or protein in mouse oocytes,

although a weak expression of *SSB-4* mRNA was apparent. This result concords with recent report of SSB-4 gene expression in oocytes at different stages of development (Pan *et al.* 2005). The ovarian granulosa cells are thought to originate from the ovarian rete cords, which are composed of cells that invaded the ovary from the overlying mesonephros during embryonic development. In contrast, the germ cells are derived from posterior embryonic ectoderm (Matsui & Okamura 2005). Thus, the oocyte and granulosa cells do not share an embryonic lineage as do the oocyte and nurse cells of *Drosophila*. The expression pattern of *SSB-1* and *SSB-4* also contrasts with other mammalian homologues of genes regulating oogenesis in flies. In these other instances, including *vasa*, *nanos*, *tudor* and *fat facets*, the mammalian homologues are also expressed in the germ cells (Fujiwara *et al.* 1994; Tsuda *et al.* 2003; Chuma *et al.* 2003; Noma *et al.* 2002). The mechanism responsible for this lineage switch in expression pattern of SSB-1 and SSB-4 is unknown, although our mRNA expression data indicate that it is controlled at the level of transcription.

SSB-1 has a predicted molecular weight of about 31 kDa and histidine-tagged SSB-1 migrates at about this position (Wang *et al.* 2005). We also observed a 31 kDa protein in HeLa and CHO cells transfected with SSB-1. Unexpectedly, these cells also showed several immunoreactive species of higher M_r that became more prominent with extended culture. Importantly, these species were not observed in non-transfected cells (Figure 5) or in cells co-transfected with SSB-1 and an siRNA targeting this mRNA (Y Xing and H.J.Clarke, unpublished observations), indicating that they require expression of SSB-1. Among these species was one of about 36 kDa that co-migrated with an immunoreactive

species detected in both primary granulosa cells and immortalized granulosa cell lines. As we have been unable to characterize these immunoreactive species, we cannot formally rule out that they represent proteins unrelated to SSB-1 that are both induced by SSB-1 and also recognized by anti-SSB-1 antibodies. A hypothesis that seems more conservative, however, is that SSB-1 can be subject to post-translational modifications. Thus, we propose that the immunoreactive species migrating at 36 kDa in granulosa cells represents a post-translationally modified form of SSB-1.

As discussed in the Introduction, GUS is required for appropriate localization of VASA and the generation of polarity in *Drosophila* eggs. There has been considerable recent interest in the possibility that mammalian eggs may also manifest a developmentally relevant polarity, although this remains highly controversial (Gardner 2001; Hiiragi & Solter 2004). MVH is expressed in both oocytes and spermatocytes, but is required only for spermatogenesis (Tanaka *et al.* 2000). Our data showing that SSB-1 and SSB-4 are not expressed in oocytes provides further evidence that an SSB/MVH-mediated polarization mechanism analogous to that of *Drosophila* does not operate in mammalian oocytes. However, we have not examined SSB expression in primordial germ cells, which express MVH (Toyooka *et al.* 2000). Thus, it is possible that SSB and MVH function together at this stage of gametogenesis.

The function of SSB-1 and SSB-4 in granulosa cells remains to be identified. Several lines of evidence suggest that SSB family proteins may physically interact with other proteins. GUS interacts with VASA, as revealed by co-immunoprecipitation experiments

(Styhler *et al.* 2002). Although the site of interaction has not been identified, the SPRY domains of other proteins mediate protein-protein interactions. In the case of SSB-1, its SPRY domain mediates its binding to the tyrosine kinase domain of the MET receptor (Wang *et al.* 2005). Moreover, SSB-1 binding enhances transcriptional activation following MET stimulation by hepatocyte growth factor. In this context, it is interesting to note that MET is expressed in granulosa cells (Parrott & Skinner 1998; Yang & Park 1995). It is tempting to speculate that SSB-1 may play a key role in regulating MET-mediated cellular responses in these cells.

Acknowledgements

Supported by the Program in Oocyte Health of the Canadian Institutes for Health Research (H.J.C.). We thank Profs. Ilpo Huhtaniemi (Imperial College London, UK) and Riaz Farookhi (McGill University) for generously supplying cell lines.

Figures

Figure 1

(A) Amino acid sequence of mouse SSB-1 (NP_083311) and SSB-4 (NP_660116) and *Drosophila* GUS (CG2944_RB). Identical residues are highlighted in red. SSB-1 and SSB-4 are about 75% identical and each about 70% identical to GUS. (B) Amino acid sequence of SSB-1 in different organisms. Identical residues are highlighted in red. Note only 4 amino acids differ between human and mouse sequences. The blue band indicates the SPRY domain, the pink band the SOCS box.

Figure 1: Xing et al

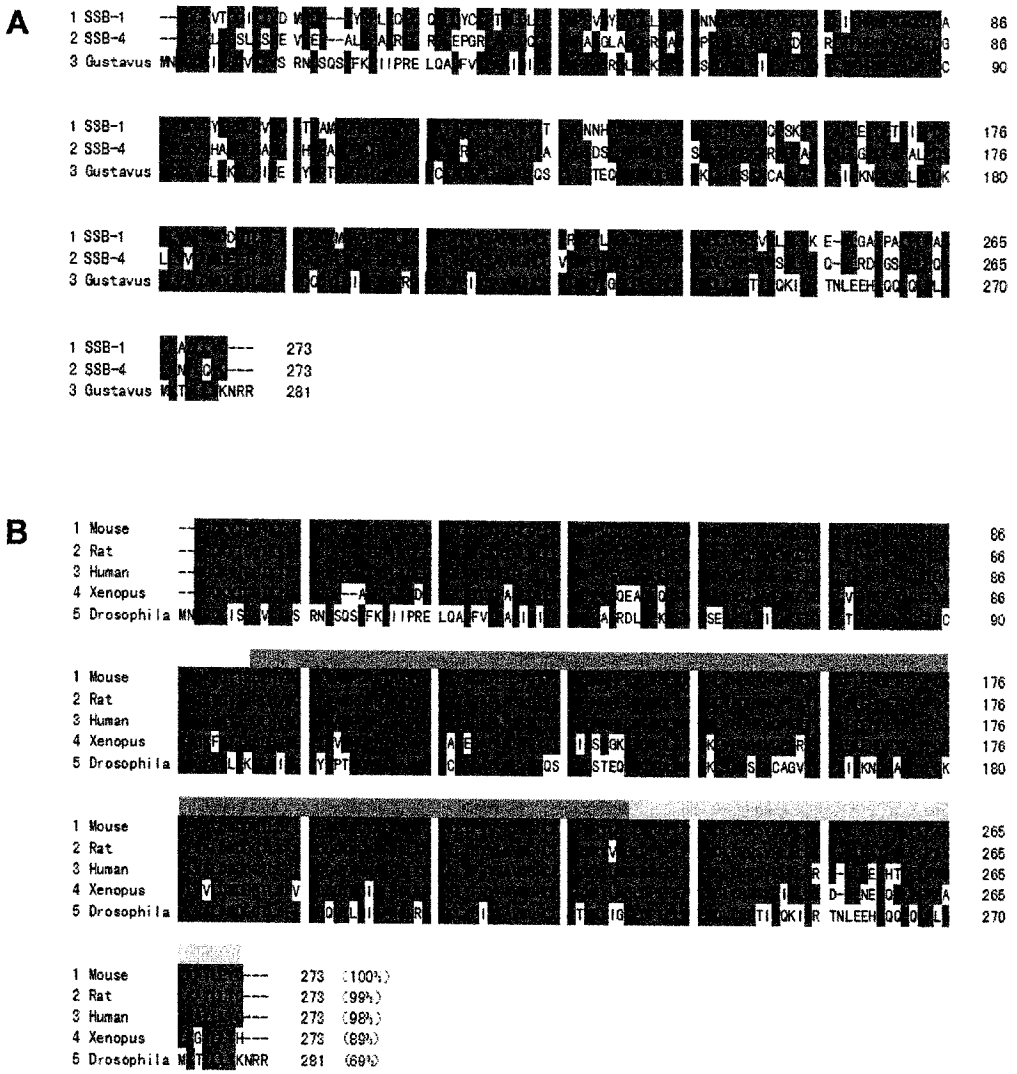


Figure 2

Analysis of *SSB-1* and *SSB-4* expression by Northern blotting. Total RNA (A) or poly(A)+ RNA (B) was analyzed using probes corresponding to *SSB-1* or *SSB-4*. The *SSB-1* probe recognizes a species of approximately 3 kb in ovary and heart and a species of approximately 1.4 kb in testis. *SSB-4* probe recognizes a species of about 2.8 kb in heart, ovary, kidney and whole embryo. Glucose-3-phosphate dehydrogenase (*G3PDH*) was used as a loading control.

Figure 2: Xing et al

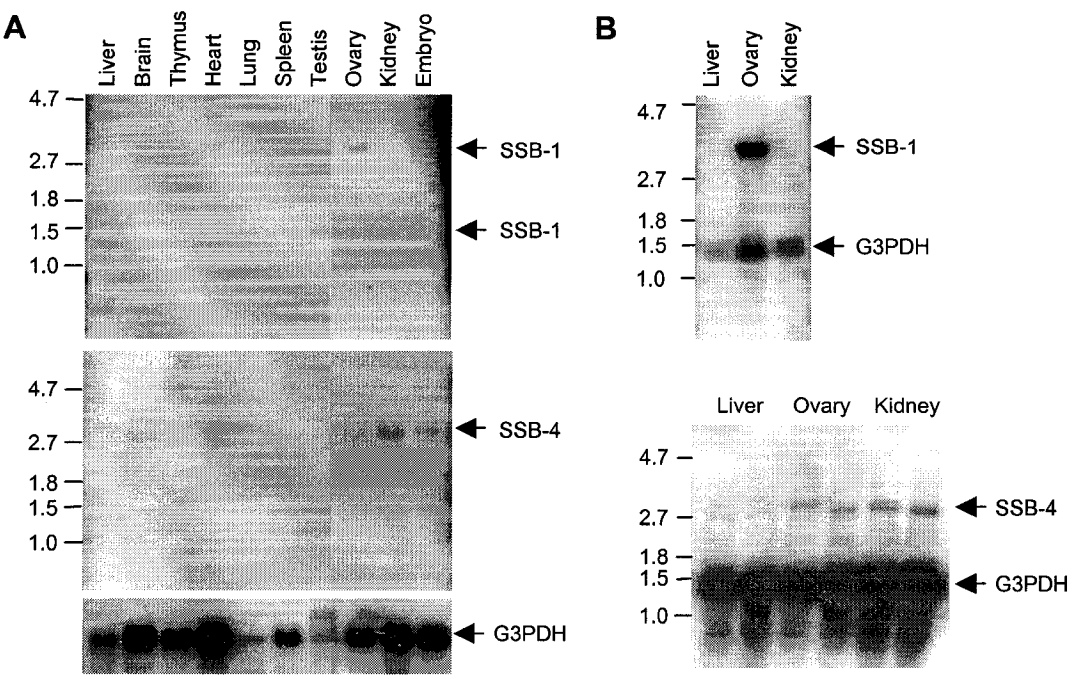


Figure 3

(A) Putative exon-intron structure of mouse *SSB-1*. Published cDNA and genomic sequences of *SSB-1* were compared to generate putative exon-intron map. Nucleotide positions are as in NT_039258.4. A putative polyadenylation sequence that may account for the truncation of exon 4 in testis is indicated. (B) Structure of *SSB-1* transcripts in ovary and testis as determined by RT-PCR using exon-specific primers and sequencing. Ovarian transcripts contain either exon 1 or exon 2 but not both. The coding sequence, indicated by textured bars, begins within exon 3 and ends within exon 4 and is identical in all transcripts.

Figure 3: Xing et al

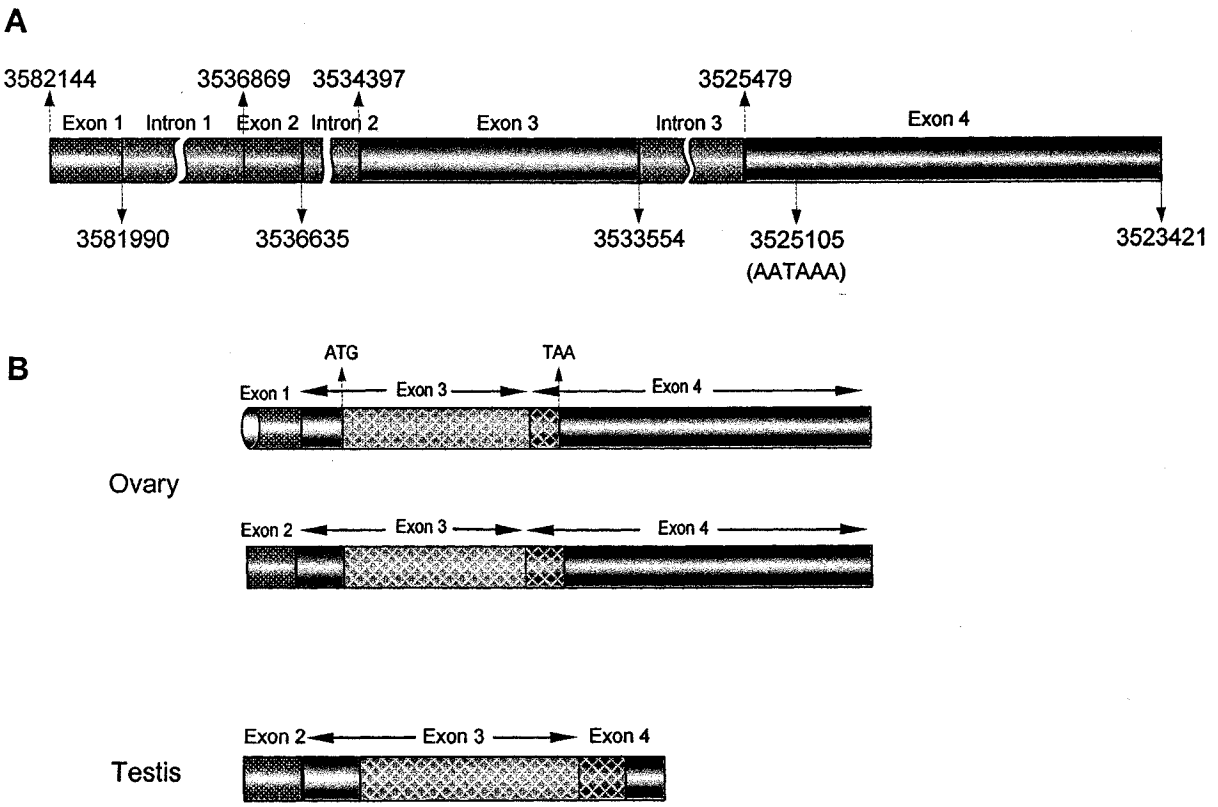


Figure 4

(A, B) Analysis of *SSB-1* (A) and *SSB-4* (B) expression by *in situ* hybridization. Ovaries were fixed in 4% *para*-formaldehyde, embedded in paraffin, and sectioned at 7-μm thickness. The right side shows results using sense-strand probes. Hybridization signals are concentrated over granulosa cells of primary and more advanced follicles. Inset in (A) shows absence of signal over oocyte. (C) Analysis of *SSB-1* and *SSB-4* expression by RT-PCR. Purified populations of granulosa cells and oocytes were collected and subjected to RT-PCR using primers corresponding to *SSB-1*, *SSB-4*, *ZP-3*, and the FSH receptor. *SSB-1* and *SSB-4* are expressed in granulosa but not detectable in oocytes. Restriction enzyme digestion confirmed that the *SSB-1* primers did not amplify *SSB-4* cDNA and vice-versa (not shown). Absence of ZP3 signal in granulosa cells confirms absence of contaminating oocytes, and absence of FSH-R signal in oocytes confirms absence of granulosa cells. Ovaries from 5 different mice for each stage were examined.

Figure 4: Xing et al

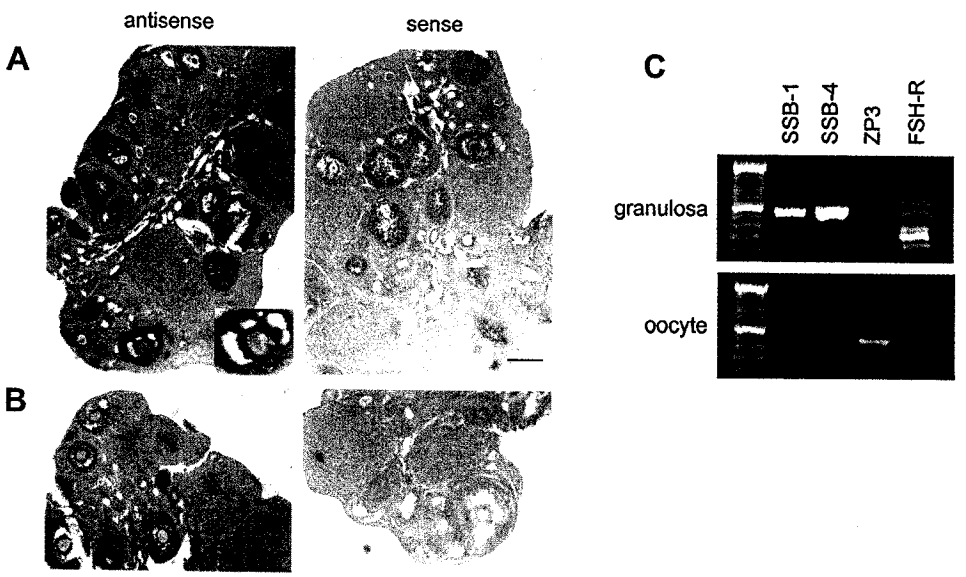


Figure 5

Detection of multiple SSB-1-related species in transfected cells. (A) CHO cells that were not transfected, transfected with a plasmid encoding SSB-1 with 100 amino acid tag, or transfected with a plasmid encoding SSB-1 were harvested 24 or 48 hr post-transfection and imunoblotted using affinity-purified anti-SSB-1. The 40 kDa band in all lanes is likely unrelated to SSB-1 (see text). SSB-1-transfected cells display a 31 kDa band 24 hr after transfection and a weaker 36 kDa band that is more intense 48 hr after transfection. Cells transfected with SSB-1 with 100 amino acid tag display a band just above 40kDa band.

(B) HeLa cells were not transfected or transfected with the construct encoding SSB-1 and blotted using affinity-purified anti-SSB-1. In addition to the 31 kDa band, several higher molecular weight immunoreactive species are present.

Figure 5: Xing et al

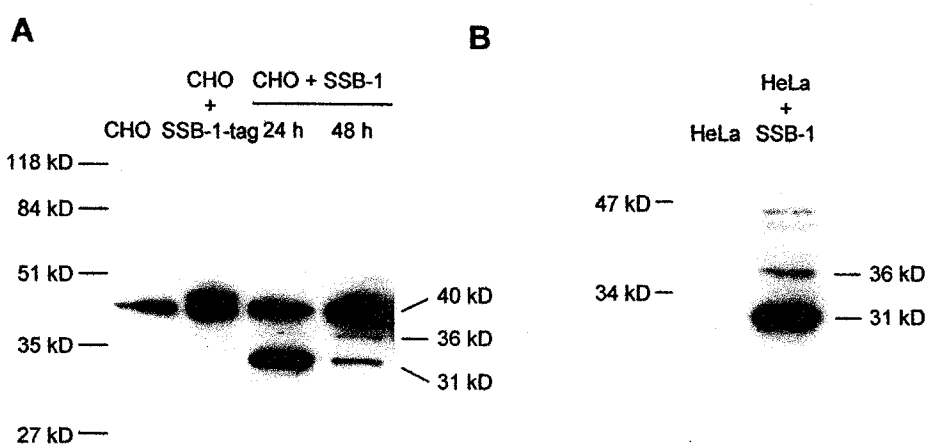


Figure 6

Detection of SSB-1 in ovarian granulosa cells and cell lines. (A) Comparison of transfected CHO cells and whole-ovary extracts blotted using anti-SSB-1. The left panel shows a long exposure of a different CHO cell blot than that shown in the right panel. Both contain the 31 kDa, 36 kDa and 40 kDa immunoreactive species. (B) Ovaries were digested using collagenase to yield granulosa-oocyte complexes or using collagenase and trypsin to dissociate the cell types. Oocytes (OO), complexes (GOC), or granulosa cells (GC) were collected and immunoblotted. The 31 kDa and 36 kDa species are present in complexes and purified granulosa cells but not in purified oocytes. (C) Extracts were prepared from whole ovaries and testes and from immortalized granulosa cell lines: NT-1 and KK-1 from mouse and DC-3 from rat. The 31 kDa and 36 kDa species are present in all groups, with the latter apparently existing as a doublet. (D) Granulosa-oocyte complexes were collected from mice at different ages, yielding populations enriched for complexes at successively more advanced stages of folliculogenesis. SSB-1 is present at all stages of follicular growth.

Figure 6: Xing et al

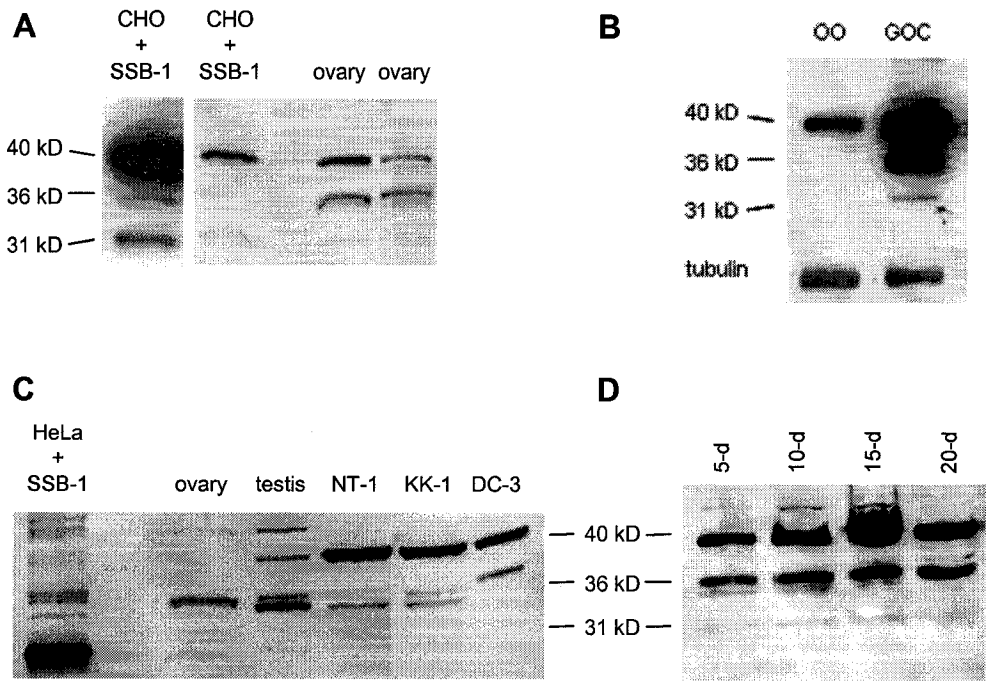
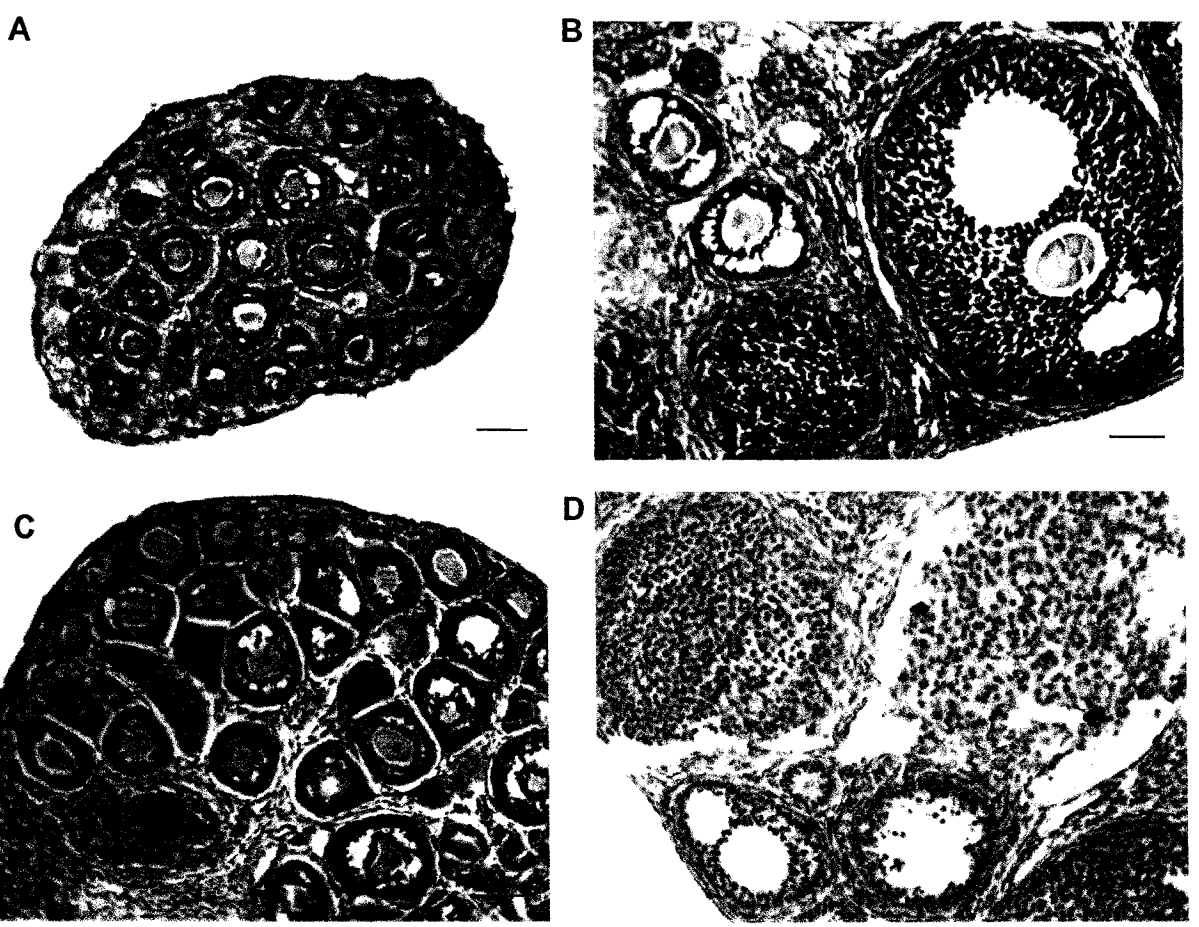


Figure 7

Detection of SSB-1 and SSB-4 by immunohistochemistry. Ovaries were fixed in 4% *para*-formaldehyde, embedded in paraffin and section at 5-μm thickness. Sections were subjected to an antigen retrieval technique. (A, B) SSB-1 is detected in granulosa cells, including both mural and cumulus of antral follicles, but not in the thecal cells or interstitial tissue. (C). SSB-4 is detected in granulosa cells in follicles at different stages of growth. (D) Sections processed using the same concentration of rabbit IgG as the primary antibody show no specific staining. Non-specific staining of oocytes is sometimes observed using this immunodetection procedure. Scale bar in A, C: 100μm. Scale bar in B, D: 50μm. Ovaries from 5 different mice was examined.

Figure 7: Xing et al



3. MANUSCRIPT II

Connecting text

Murine homologues of the Gustavus gene of Drosophila, SSB-1 and SSB-4 are expressed in mouse ovarian granulosa cells. As well, SSB-1 and SSB-4 mRNA are expressed in mouse testis tissue. Sertoli cells in the testis come from the same embryonic precursor as granulosa cells in the ovary and play an analogous role in supporting germ cell development. Most importantly, GUS has been identified as a VASA-interacting protein in Drosophila. Murine VASA homologue (MVH) has been shown to be expressed in male germ cells, and deletion of this gene leads to male sterility. We therefore tested the hypothesis that SSB-1 is expressed both in male germ cells and in Sertoli cells.

Abstract

Murine homologues of *Drosophila* Gustavus, SSB-1 and SSB-4, are expressed in mouse ovarian granulosa cells at all stages of folliculogenesis. We investigated the expression pattern of SSB-1 in mouse testis tissue. Highly purified populations of male germ cells at different stages of spermatogenesis were obtained by cellular sedimentation. Results of immunoblotting revealed that SSB-1 was strongly expressed in the spermatocytes and round spermatids but weakly in elongated spermatids. We also tested the expression of SSB-1 in Sertoli cells, because these cells are derived from the same embryonic precursors as the granulosa cells and, like the granulosa cells, play an essential role in supporting germ cell differentiation. Results of immunoblotting revealed that SSB-1 was detectable in three immortalized Sertoli cell lines, TM4, MSC-1 and SF7. Immunostaining analysis of mouse testis sections revealed that SSB-1 is localized in the cytoplasm of the germ cells. The results of whole-mount staining of germ cells showed that SSB-1 was localized in the chromatoid body in round spermatids. These results reveal that SSB-1 protein is expressed in mouse Sertoli cells and male germ cells. Intriguingly, like mouse VASA homologue (MVH), it is localized in chromatoid body. Gene products found in this structure have been demonstrated to play important function in post-transcriptional RNA processing. Therefore, SSB-1 might, like MVH, play a crucial function in mouse spermatogenesis.

Introduction

The differentiation of the germ cells, like other cell types, is a highly complex and ordered process. Understanding the genetic control of this process will provide fundamental insight into mechanisms of cellular differentiation and the concept of cellular totipotency and is crucial for improving our ability to treat human infertility. Over the past few years, a number of genes have been identified as playing key roles in gametogenesis, from development of the primordial germ cells through differentiation of mature oocytes and sperm that are competent to support development of a new individual (Zhao & Garbers 2002; Brennan & Capel 2004; Park & Jameson 2005; Roy & Matzuk 2006).

Such studies have identified a number of genes for which an important function during gametogenesis has been conserved during evolution. One example is *nanos*, which was identified in *Drosophila* as a gene whose protein product is present in germ cells throughout development (Forbes & Lehmann 1998). Early work showed that the *nanos* gene product, a RNA-binding protein that acts as a translational repressor, was required for the migration and survival of primordial germ cells. Recent studies have revealed an additional role for NANOS; it prevents differentiation of female germ-line stem cells into oocytes (Wang & Lin 2004). Three homologues of *nanos* have been identified in mice. *Nanos2* is predominantly expressed in male primordial germ cells, and mutants lacking the gene show complete loss of male germ cells by the time of birth. In contrast, female gonads are morphologically normal and homozygous-null females are fertile. In the absence of *Nanos3*, however, primordial germ cells are lost from both males and females

during early embryogenesis, apparently even before reaching the primitive gonad. Thus, members of the *Nanos* gene family are required in mice, as in flies, for early germ cell development.

The *DAZ* family of genes provides another example of conserved activity during gametogenesis. Four DAZ genes are found on the human Y-chromosome (Reijo *et al.* 1995) and related autosomal genes are also present. Homologues have been identified in numerous organisms (Xu *et al.* 2001). Human *Daz* is expressed in male but not female germ cells and its deletion causes male infertility (Xu *et al.* 2001). In mice, which do not contain Y-encoded *Daz* genes, deletions of the autosomal *Dazl* gene causes sterility in both males and females. Germ cell loss has been reported to occur shortly after entry into meiosis in both males and females (Saunders *et al.* 2003), although recent results indicate an earlier loss in males on an inbred genetic background (Lin & Page 2005). In *Drosophila*, loss of the *Daz* homologue, *Boule*, causes meiotic arrest in males (Eberhart *et al.* 1996). Thus, *Daz* genes are required for male germ cell development in numerous species.

Mouse vasa homologue (*Mvh*) is related to the *vasa* gene of *Drosophila*. *Mvh* is expressed in the germ cells of both sexes (Fujiwara *et al.* 1994; Toyooka *et al.* 2000). However, its function seems to be limited to males, as deletion of the gene leads to male infertility whereas females show no defects (Tanaka *et al.* 2000). In males, MVH is detectable throughout spermatogenesis up to post-meiotic spermatids and, when it is absent, germ cells fail to progress beyond the pachytene stage. Within the cell, MVH is restricted to the

cytoplasm and is localized with a structure known as the chromatoid body. This is an electron-dense structure found adjacent to the nucleus (Parvinen 2005). It has been identified at different stages of spermatogenesis (Parvinen 2005), though some researchers consider the definitive chromatoid body to be restricted to spermatids (Kotaja *et al.* 2006). Although known to exist for many years, its function remains poorly understood. It is thought to contain RNA and, based in part on the presence of MVH, which has RNA helicase activity, to play a role in RNA storage and processing (Kotaja *et al.* 2006). However, its molecular composition is largely unknown.

Gus was recently identified as a gene required for oogenesis in Drosophila (Styhler *et al.* 2002). GUS physically interacts with VASA in oocytes of the fly, suggesting that the two proteins are functionally linked. We recently identified the mouse genes *SSB-1* and *SSB-4*, as potential homologues of *gus* (Xing *et al.* 2006). In contrast to *gus*, however, neither *SSB-1* nor *SSB-4* is expressed at detectable levels in mouse oocytes. In view of the interaction between GUS and VASA that has been established in the fly, together with the demonstrated role of MVH during spermatogenesis in the mouse, we therefore examined whether *SSB-1* and *SSB-4* were expressed during spermatogenesis in the mouse.

Materials and Methods

Animals

CD-1 outbred mice were purchased from Charles River Canada (St-Constant, QC).

Cell culture and transfection conditions

Mouse Sertoli cell lines SF7, MSC-1 and TM4 were generously supplied by Prof. M. Nagano (McGill University, Canada). NT-1 cells were generously supplied by Prof. I. Huhtaniemi (Imperial College, London, UK). HeLa cells and 293T cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM, or DMEM-F12 for NT-1 cells, supplemented with 10% fetal bovine serum.

Isolation of purified populations of male germ cells

Male germ cells were isolated from the testes of 10 mice aged 70 days by cellular sedimentation at unit gravity on a 2-4% BSA gradient using the STA-PUT apparatus as previously described (La & Trasler 2006). Purities of recovered cells were determined using morphological criteria and were 70% for pachytene spermatocytes, 92% for round spermatids and 98% for elongating spermatids mixed with residual bodies.

Cell transfection

The protein-coding region of SSB-1 was inserted into pTriEX-2 (Novagen, CA,USA) and sequenced to confirm its identity. Cells were transfected when they had reached 70% confluency. Four μg of plasmid and 10 μl of Lipofectamine 2000 (Invitrogen, Burlington, ON) were incubated separately in 250 μl of serum-free medium for 5 min, combined and

allowed them to stand for 20 min, then added drop-wise to the plate containing the cells. The culture medium was changed 6 hours after transfection and the cells were harvested by trypsinization 24 hours after transfection.

Antibodies

Rabbit antibodies recognizing SSB-1 and SSB-4 were prepared using as immunogens peptide sequences that were unique to each protein and did not include the SOCS box or SPRY domain (SSB-1 sequence: N 15-31: DPTYRPLKQELQGLDYC ; SSB-4 sequence: N 16-32: EPALRPAKRELRGLEPG). Antibodies were prepared and affinity-purified by a commercial supplier (Biosynthesis, TX, USA).

Immunoblotting

Mouse testes were rapidly homogenized in 2X gel-loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.1% bromophenol blue, 10% ß-mecaptoethanol), denatured at 95°C for 5 min, and centrifuged to pellet the debris. The cell lines were harvested using 0.25% trypsin-EDTA (0.025g/L Trypsin, 0.2g/L EDTA.Na), then centrifuged at 500g for 5 min. The supernatant was discarded and the pellets were dissolved in loading buffer and denatured as above. Protein electrophoresis, transfer to PVDF membranes and immunoblotting were carried out as previously described (Xing et al., 2006). Primary antibodies were used at a dilution of 1:1000; secondary antibodies conjugated to horseradish peroxidase (HRP, Promega, Montreal, QC, Canada) at a concentration of 1:5000. HRP activity was revealed using ECL+ (Amersham, Montreal, QC, Canada) following the manufacturer's directions.

Whole-mount seminiferous tubule preparations

Seminiferous tubules were prepared as described (Kotaja *et al.* 2006) Briefly, mice were sacrificed and the testes removed and decapsulated. The seminiferous tubules were transferred to a dish containing PBS and gently pulled apart using forceps. For further analysis, a small fragment (about 0.5 mm) of the testis was cut transferred to a glass microscope slide in 15 µl of PBS and covered with a cover slip. The weight of the cover slip causes the spermatogenic cells to flow out of the tubule. For fixation, the slides were plunged in liquid N_2 to freeze them, the cover slip was flipped off, and the cells stuck to the slide were fixed for 5 minutes using 90% ethanol.

Immunostaining

Testes were excised and fixed overnight at 4°C in freshly prepared 4% *para*-formaldehyde, washed in PBS, dehydrated through ethanol and xylene, and embedded in paraffin. Five-micron sections were cut and mounted on glass microscope slides. Slides were exposed to 3% H_2O_2 in methanol for 10 minutes to quench endogenous peroxidase activity, then washed 3 times in H_2O. They were placed in an antigen retrieval solution (Xing *et al.* 2006) and boiled for 10 min. After cooling, the slides were washed 3 times in PBS containing 0.1% Tween-20 (PBST). They were treated with a blocking solution (PBST, 3% bovine serum albumin) for 1 hour, then incubated overnight at 4°C with affinity-purified anti-SSB-1 or anti-SSB-4 diluted 1:200 in blocking solution. After three 30 min washes in PBST, in some experiments, the slides were incubated with fluorochrome-conjugated secondary antibodies (Jackson Immunoresearch, Molecular

Probes) for 1 hour or then mounted in glycerol. DNA was counterstained using DAPI (1 μg/ml) or propidium iodide (10 μg/ml) added to the mounting medium. Slides were observed under fluorescent microscope (Leica) or confocal microscope (Zeiss CSLM). In other experiments, sections were incubated in biotinylated secondary antibodies (ABC kit, Vector Laboratories, Burlington, ON, Canada) for 1 hour, washed and incubated with the ABC complex following the manufacturer's instructions. The sections were washed and stained with AEC color substrate (Vector Laboratories, CA, USA), then washed and counter-stained with hematoxylin for 30 sec. Slides were mounted in glycerol and observed under a bright-field microscope.

Results

SSB-1 and SSB-4 proteins are expressed in male differentiating germ cells

Results of Northern blotting indicated that mRNA encoding SSB-1 and SSB-4 were expressed in the testis (Xing *et al.* 2006). To examine whether SSB-1 and SSB-4 proteins were expressed in testes, we first prepared testis extracts and blotted these using the anti-SSB-1 antibody. As shown in Figure 1A, three major immunoreactive bands were observed. These co-migrated with the principal bands detected in ovarian extracts, except that the ~36 kDa species appeared to exist as a doublet, with the more abundant species migrating slightly faster than the single species detected in ovaries. This suggests that both SSB-1 and the same immunologically related proteins are present in both organs.

To identify the cell types expressing SSB-1, we then prepared purified populations of germ cells at different stages. Because the spermatogenic cells become progressively smaller during the two meiotic divisions and spermiogenesis, purified populations at different stages of spermatogenesis can be obtained simply by applying a single-cell testicular suspension to the top of a dense solution, allowing the cells to sediment through the solution at different rates depending on their size, and collecting the fractions. Although this technique does not yield large populations of cells at all different stages of spermatogenesis, we were able to obtain highly purified populations of primary spermatocytes, round spermatids and elongating spermatids.

As shown in Figure 1B, the 31 kDa species was present in primary spermatocytes and round spermatids, whereas it was only weakly detectable in elongating spermatids. In

contrast, the 36 kDa doublet was present in all three cell types, with the faster-migrating species being considerably more prominent. The 36 kDa immunoreactive species was more prominent in testis as compared to ovary and also migrated slightly more rapidly whereas the 31 kDa species was relatively reduced.

The two methods were used to test whether the other immunoreactive species might be glycosylated forms of SSB-1. Ovarian tissue extracts were treated with N-Glycosidase F, also known as PNGase, but this did not change the electrophoretic migration of the 40 and 36 kDa bands. Next, the rat granulosa cell line, DC3, was treated with with tunicamycin, an inhibitor of glycosylation. As above, no effect on the mobility of the 40 and 36kDa bands could be detected (data not shown). We concluded that the 40 and 36kDa immunoreactive species are not glycosylated forms of SSB-1.

To examine the intracellular localization of SSB-1, histological sections of testes were prepared and stained using the anti-SSB-1 antibody (Figure 2). During spermatogenesis, the differentiating germ cells move from the basal (exterior) region of the seminiferous tubule to the luminal (interior) region where mature sperm are released into the lumen. The spermatogonia and, in much smaller numbers, the Sertoli cells are found basally, the spermatocytes lie luminal to the spermatogonia, and the spermatids and mature sperm constitute the most luminal cell populations.

In testicular sections stained using anti-SSB-1, three populations of cells can be distinguished within the seminiferous tubules (Figure 2A, B). The cells in the basal layer

were only weakly stained. Luminal to this was a broad layer of cells showing strong cytoplasmic staining. The luminal-most cells were weakly stained. This suggests that the anti-SSB-1 most strongly stained the spermatocytes, whereas spermatogonia (basal) and spermatids were weakly stained. Although the antibody recognizes several bands in immunoblots in addition to the 31kDa species that co-migrates with SSB-1, these results are consistent with the expression profile of SSB-1 revealed using immunoblotting. Similar results were obtained when sections were stained using anti-SSB-4 (Figure 2C). Figure 2A also shows bright staining of cells lying between the seminiferous tubules; however, this was also observed using the secondary antibody alone. These results suggest that SSB-1 and SSB-4 are abundantly expressed in the cytoplasm of spermatocytes and less so in spermatids.

Mouse vasa homologue (MVH) has recently been identified in the chromatoid body of spermatogenic cells. Since, in the fly, GUS physically interacts with VASA, and MVH is found in the chromatoid body, we wished to test whether SSB-1 might also be associated with the chromatoid body. We produced whole-mount preparations of spermatogenic cells and stained these using anti-SSB-1. The chromatoid body could be identified lying adjacent to the nuclei of spermatids (Figure 3). It was weakly stained in dried spermatids but prominently stained using a whole-mount technique in which the cytoplasm is retained. These results are consistent with the hypothesis that SSB-1 and MVH are co-localized in the chromatoid body of spermatids. Nevertheless, this remains to be fully established, as the presence of multiple bands in immunoblots probed using anti-SSB-1 means that we cannot be certain that the chromatoid staining reflects the presence of SSB-

1. Moreover, we have been unable to co-immunoprecipitate these proteins from testis extracts using either anti-SSB-1 or anti-MVH as the precipitating antibody (data not shown).

SSB-1 protein is expressed in Sertoli cells

SSB-1 protein is expressed in the granulosa cells of the ovary, although it is undetectable in oocytes (Xing *et al.* 2006). The Sertoli cells of the testis are derived from the same embryonic precursors as the granulosa cells and, like the granulosa cells, play an essential role in supporting germ cell differentiation. Therefore, we speculated that SSB-1 might also be expressed in Sertoli cells. Because Sertoli cells make up only a small fraction of the cells in the testis, it is not feasible to collect large purified populations. We therefore analyzed SSB-1 expression in immortalized cell lines derived from Sertoli cells.

TM4 cells were derived from the testis of an immature BALB/c mouse (Mather 1980). MSC-1 cells were established from transgenic mice carrying a fusion gene composed of human Mullerian inhibitory substance transcriptional regulatory sequences linked to the SV40 T-antigen gene (McGuinness *et al.* 1994). SF7 cells were immortalized using the SV40 large T antigen (Hofmann *et al.* 1992). We found that, when immunoblotted using the anti-SSB-1 antibody, three mouse Sertoli-derived cell lines, as well as the granulosa-derived cell line, NT-1, expressed the same three protein species (Figure 1). Therefore, SSB-1 protein is expressed in the Sertoli cells as well as in the germ cells of male mice.

Discussion

We have examined the expression and intracellular localization of SSB-1 in the testis of the mouse. We find that SSB-1 is immunologically detectable in extracts prepared from whole testis. It was detectable by immunoblotting in cell populations enriched for primary spermatocytes and spermatids. Immunofluorescent analysis revealed a strong staining in spermatocytes as well as a weaker staining in spermatids. Using a whole-mount technique, immunoreactive species were associated with the chromatoid body of spermatids. Finally, SSB-1 was also detectable in three independent cell lines derived from Sertoli cells. These results indicate that SSB-1 is expressed in the germ cells and Sertoli cells in the testis and that, in spermatids, a proportion of the SSB-1 is found in the chromatoid body.

The observation that SSB-1 is present in testis is consistent with our earlier observation that the encoding mRNA was present (Xing *et al.* 2006). Thus, SSB-1 protein is in both ovary (Xing *et al.* 2006) and testis. Nonetheless, differences in the protein may exist between these two tissues. Our results indicated that the 36 kDa immunoreactive species was more prominent in testis as compared to ovary and also migrated slightly more rapidly (see Figure 1), whereas the 31 kDa species was relatively reduced. The relationship between these two immunoreactive species is not known. However, both were reduced in immortalized granulosa cells treated with short interfering (si) RNA targeting *SSB-1* (see following chapter), implying that both are products of this gene.

Post-translational modifications such as phosphorylation may underlie the differences in electrophoretic mobility.

The observation that SSB-1 is present in male germ cells is intriguing in light of the previous reports that MVH is also present in these cells and that, when MVH is absent, spermatogenesis is compromised. Thus, male germ cells express both MVH and SSB-1, and MVH is required for their development, whereas female germ cells express only MVH, and it is not required for their development. In flies, GUS and VASA physically interact, as indicated by co-immunoprecipitation studies using ovarian lysates (Styhler *et al.* 2002). Based on these data, it may be proposed that GUS and VASA (in the fly) and SSB-1 and MVH (in the mouse) regulate events that are required for oogenesis in flies and spermatogenesis in mice. These events appear not to be required, however, for oogenesis in the mouse.

Although the role of SSB-1 and MVH during spermatogenesis remains unknown, their co-localization in the chromatoid body is intriguing. In view of the fact that GUS and VASA physically interact in the fly, it could be proposed that SSB-1 and MVH interact in the chromatoid body. In support of this notion, RanBPM, a microtubule-binding protein that contains a SPRY domain, has recently been shown to be present in the chromatoid body and to co-immunoprecipitate with MVH (Shibata *et al.* 2004). Both RanBPM and SSB-1 contain a SPRY domain, and this is known to mediate interaction of SSB-1 with other proteins. It may be speculated that the SPRY domain mediates interaction of proteins, including SSB-1 and RanBPM, with MVH.

Recent work indicates that the chromatoid body may serve as a site of mRNA processing, possibly including opposing processes such as storage and degradation. Notably, several components of the microRNA pathway have very recently been identified in the chromatoid body (Kotaja *et al.* 2006). As VASA is thought to translationally regulate several mRNAs, its localization in the chromatoid body is consistent with this link to mRNA metabolism. Thus, SSB-1 may be required for proper MVH-regulated translational control of mRNAs localized within the chromatoid body.

Sertoli and ovarian granulosa cells express numerous genes in common, such as the follicle-stimulating hormone receptor, prolactin receptor, and aromatase. Therefore, it is not unexpected that SSB-1, which is prominently expressed in granulosa cells, is also expressed in Sertoli cells. Its function in these cells remains to be identified. One way to address this might be through the use of mice expressing the Cre recombinase gene linked to the promoter of the anti-Mullerian hormone gene. Such mice have been used to selectively remove the androgen receptor from Sertoli cells (Chang *et al.* 2004; De Felici *et al.* 2004b; Roy & Matzuk 2006). Mice containing a region of SSB-1 exon 3 flanked by loxp sites could be made and mated with the Cre-transgenic mice to inactive SSB-1 in the Sertoli cells. In any case, future work will need to address the intracellular events regulated by SSB-1.

Acknowledgements

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Figures

Figure 1

(A) SSB-1 protein was detected by immunoblotting using affinity-purified anti-SSB-1 in the indicated cells or tissues. HeLa cells were transfected with DNA encoding SSB-1. SF7, MSC-1, TM4 are cell lines derived from mouse Sertoli cells. NT-1 cells were derived from mouse ovarian granulosa cells. The 31 kDa, 36 kDa and 40 kDa immunoreactive species were detected in all cases.

(B) SSB-1 protein was detected by immunoblotting using affinity-purified anti-SSB-1 in non-transfected and SSB-1-transfected 293T cells, in extracts prepared from whole ovary and whole testis, and in populations of male germ cells enriched for the indicated stages. Tubulin was used as loading control. The 31 kDa band is detectable in pachytene spermatocytes and round spermatids, but only weakly in elongated spermatids.

Figure 1

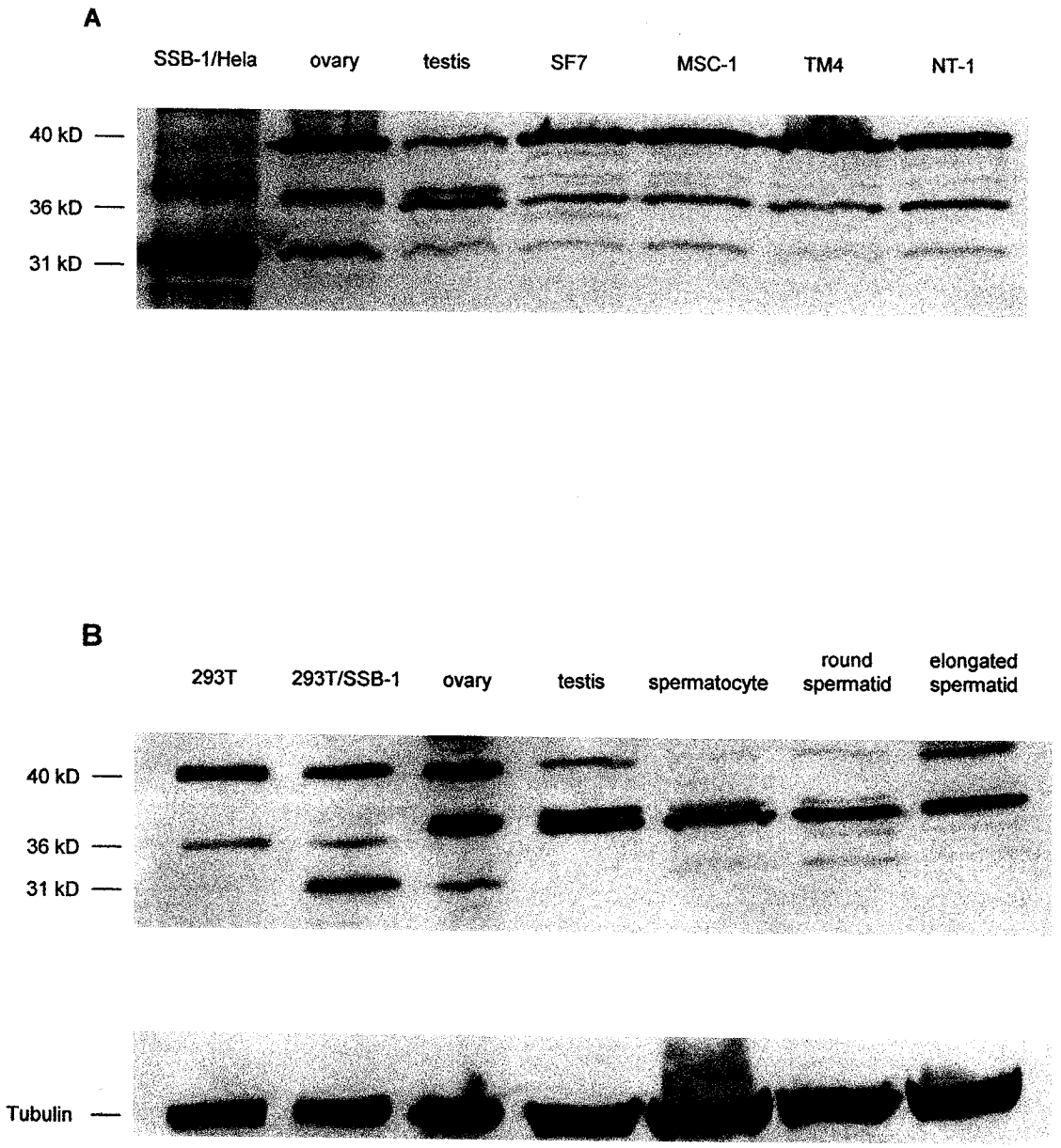

A
SSB-1/Hela
ovary
testis
SF7
MSC-1
TM4
NT-1
40 kD
36 kD
31 kD
B
293T
293T/SSB-1
ovary
testis
spermatocyte
round spermatid
elongated spermatid
40 kD
36 kD
31 kD
Tubulin

Figure 2

Sections of mature testis were immunostained with anti-SSB-1or anti-SSB-4 antibodies. Mouse adult testis were fixed in 4% *para*-formaldehyde, embedded in paraffin and sectioned at 5-μm thickness. Sections were subjected to an antigen retrieval technique then stained using affinity-purified anti-SSB-1 (A, B) or anti-SSB-4 (C). (A) Staining was detected in the cytoplasm of spermatocytes and less strongly in round spermatids. Staining between seminiferous tubules in A was also detected when the primary antibody was omitted. Scale bar: 100μm (B) Higher magnification showing absence of staining from spermatogonia and presence of cytoplasmic staining in spermatocytes and spermatids. a. SSB-1 staining. b. DNA staining using propidium iodide. c merged images. Scale bar: 10 μm. Testes from 5 different mice were examined.

Figure 2

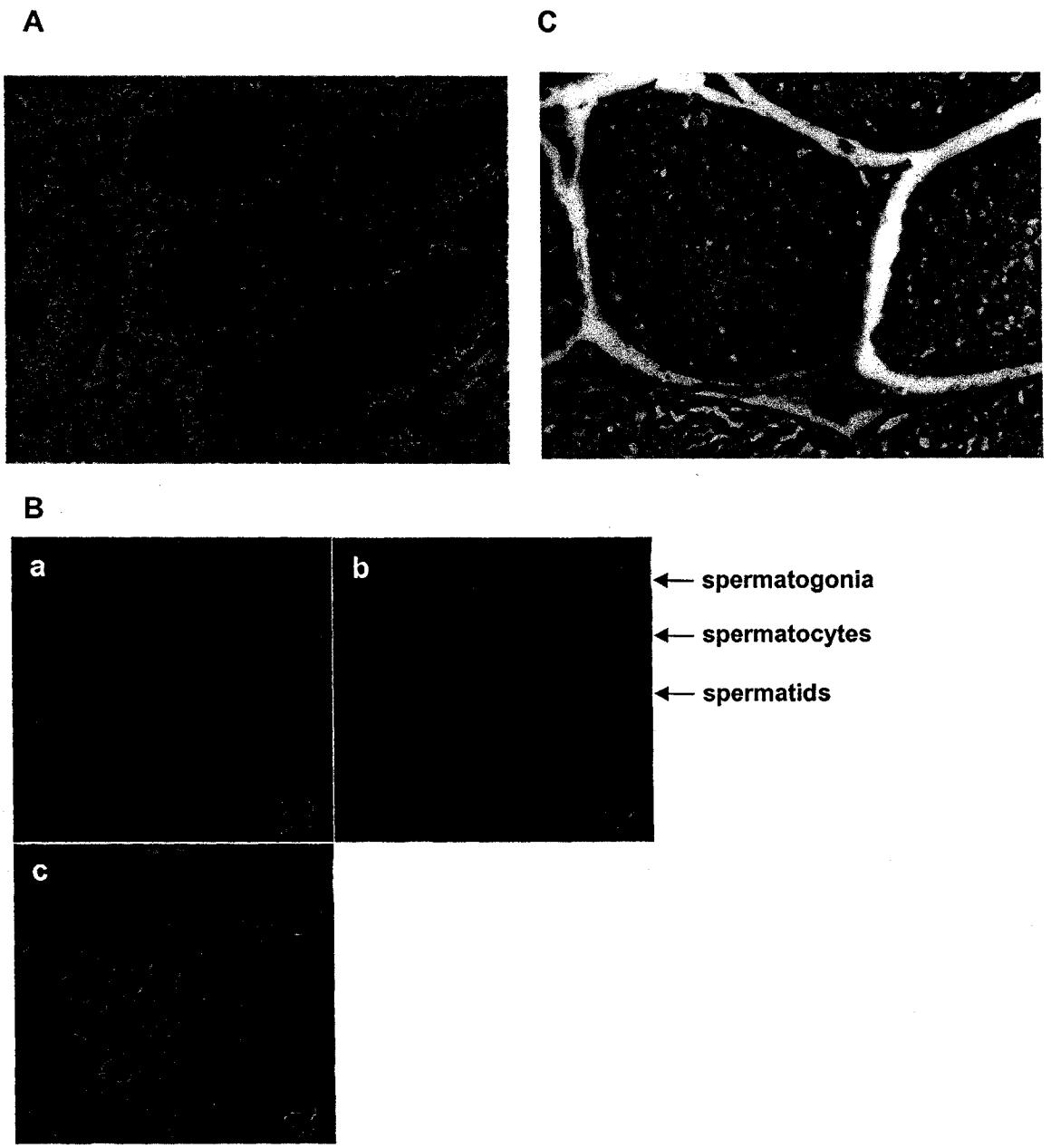
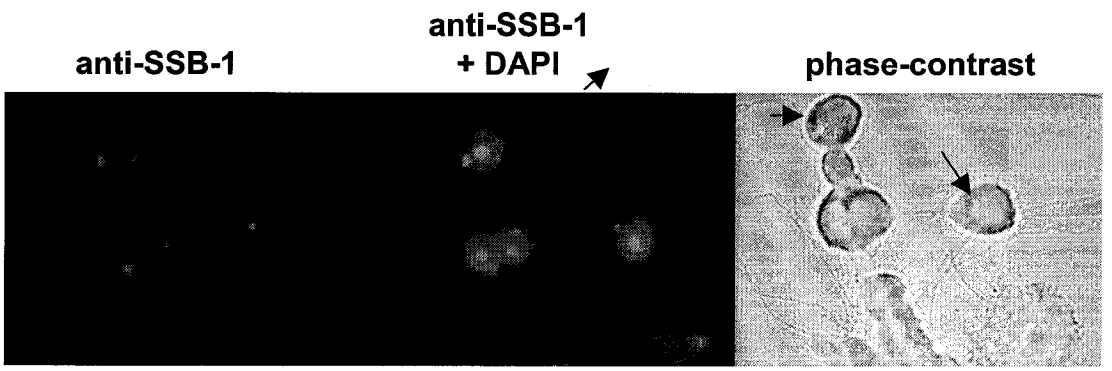


Figure 3

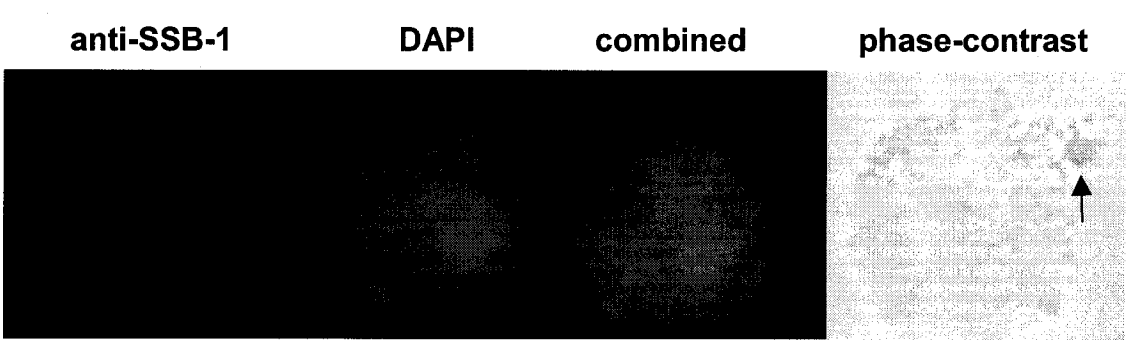
SSB-1 is a component of chromatoid body in spermatids. Anti-SSB-1 was used for immunostaining. (A) Using the whole-mount preparation technique, which retains the cytoplasm, prominent staining of the chromatoid body (arrow) is evident. Cells are identified as spermatids by the prominent focus of DNA staining near the middle of the nucleus. (B) Using a drying-down technique, in which the cytoplasm is lost, weak staining of the chromatoid body (arrow) is detectable.

Figure 3

A


anti-SSB-1
anti-SSB-1
+ DAPI
phase-contrast

B


anti-SSB-1
DAPI
combined
phase-contrast

4. MANUSCRIPT III

Connecting text

The previous results demonstrated the expression pattern of SSB-1 in mouse ovary and testis. In the ovary, SSB-1 is found in the granulosa cells at all stages of folliculogenesis. In the testis, it is present in Sertoli cells and cytoplasm of spermatocytes - most interestingly, in the chromatoid body. Here, I investigated the function of SSB-1 by depleting it from cells. Making mutant mice that do not express SSB-1 is a promising approach; however, it is time-consuming and labour-intensive, and achieving cell-type-specific depletion may be difficult because there is no Cre mice for all different stages of granulosa cells are available. Therefore, I examined the potential of the RNA interference technique to study the function of SSB-1 in ovarian granulosa cells.

Abstract

We have found that murine homologues of *Drosophila* GUSTAVUS, SSB-1 and SSB-4 are expressed in ovarian granulosa cells at all stages of folliculogenesis. To examine the fnction of SSB-1, short interfering RNA (siRNA) targeting the mRNA was transfected into primary granulosa cells within granulosa-oocyte complexes and into an immortalized granulosa-derived cell line, NT-1. Although siRNAs could be efficiently introduced into the primary granulosa cells, as assayed using flow cytometry, the amount of SSB-1 mRNA could not be decreased significantly. SSB-1 mRNA and protein could be efficiently depleted in NT-1 cells, however, declining to about 20% of cells transfected with a control siRNA. Time course experiments revealed that SSB-1 RNA and protein began to increase beyond 48 hr after SSB-1 siRNA transfection, reaching nearly the original level at 5 days after transfection. Depletion of SSB-1 led to a decrease of the protein level of P450scc, the rate-limiting enzyme in granulosa cell steroidogenesis, to about 40% of controls. MRNA levels increased beyond 48 hours after SSB-1 RNA silencing, paralleling the increase in SSB-1. These results establish that SSB-1 protein can be depleted using RNAi technique in mouse granulosa cell line NT-1; therefore, this cell line can be used to study the function of SSB-1. Moreover, they imply a potential role for SSB-1 in granulosa cell steroidogenesis.

Introduction

We previously reported that murine homologues of the *gus* gene of *Drosophila, SSB-1* and *SSB-4,* are expressed in ovarian granulosa cells (Xing *et al.* 2006). In order to know the function of those genes, the most straightforward method is to make mutant mouse that do not express the protein. Neither *SSB-1* nor *SSB-4* is expressed specifically in granulosa cells, however; rather they both are expressed in several tissues in the embryo. Therefore, such a 'knock-out' might be embryo-lethal.

A Cre-loxP strategy often is used to knock out gene expression in a specific cell type. In this procedure, a mouse carrying a gene encoding Cre recombinase under the control of a cell-specific promoter is crossed with a mouse in which the target gene has been flanked by *loxp* sites. The recombinase catalyzes recombination between the two sites, thus excising the enclosed gene, and the cell-specific expression of the Cre recombinase gene limits the excision and gene product to that cell type. But *SSB-1* and *SSB-4* are expressed in granulosa cells in follicles at all stages of development, and no mouse strains expressing *Cre* at all these stages is available so far (Roy & Matzuk 2006). Moreover, as SSB-1 and SSB-4 share 75% protein similarity, they may be functionally redundant, meaning that knocking out only one would not lead to a loss of function. Therefore, making knock-out mice to investigate the physiological significance of SSB-1 and SSB-4 in the ovary is not very practical at this stage.

RNA interference (RNAi) is a technique that has become widely used over the last few years to study gene function (Sandy *et al.* 2005). Its effectiveness is based on the

phenomenon that double-stranded RNA molecules can associate with an mRNA of matching sequence, leading either to the translational repression or degradation of the mRNA. Hence, it is a strategy to reduce or eliminate the amount of gene product in cells. This phenomenon was first identified in plants and was then shown to function in worms (Jorgensen *et al.* 1996; Cogoni & Macino 2000). It was initially thought not to have potential as an experimental tool in mammalian cells, as the presence of long double-stranded RNA (greater than 50bp) in mammalian cells will induce an anti-viral reaction that leads to a non-specific reduction in total protein synthesis (Lee & Esteban 1994; Billy *et al.* 2001). It was subsequently found, however, that the mechanism of RNAi involves the cleavage of the long dsRNA into short duplexes. Introducing these short duplexes into cells specifically suppressed the expression of the target gene (by acting on the stability or translation of the mRNA) without triggering the anti-viral response (Sayda et al, 2001). This key conceptual advance has enabled RNAi to be used as a tool to study gene function in mammalian cells. Indeed, since the first publications on siRNA about five years ago, there has been a tremendous amount of work on siRNA silencing published on different mammalian cells, tissues and organs. Compared to siRNA silencing, the traditional knockout mice strategy is slow and expensive. Therefore, we attempted to use RNAi to investigate the function of SSB-1 on ovarian granulosa cells.

Materials and Methods

Animals

Experiments were carried out using CD-1 mice (Charles River, St-Constant, Quebec, Canada). All animal procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the McGill University Health Centre.

siRNA sequence for EGFP and SSB-1

siRNA sequences were obtained from Ambion (Austin, TX, USA). These were supplied as 20 nmol in powder. Upon receipt, they were dissolved in 400 µl RNase free H_2O and divided into aliquots of 8 µl, and stored at –80°C until use. Under these conditions, the siRNAs retained their activity for at least 1 year.

Sequences were as follows:

SSB-1:

#178180: Sense: GCUGUAUCCUGUAGUGAGUTT (5'-3')

Antisense: ACUCACUACAGGAUACAGCTT (5'-3')

#83484: Sense: GGAAGAUGACAAGUUGAUCTT (5'-3')

Antisense: GAUCAACUUGUCAUCUUCCTT (5'-3')

EGFP (Lewis *et al.* 2002):

Sense: GACGUAAACGGCCACAAGUUC (5'-3')

Antisense: ACUUGUGGCCGUUUACGUCGC (5'-3')

Positive control GAPDH siRNA (Ambion #4624)

SiRNA was labelled by cy3 with siRNA Labelling kit (Ambion, Texas, USA).

Collection and culture of granulosa-oocyte complexes

Oocyte-granulosa complexes were isolated from ovaries of 12-day-old CD-1 mice as described (Eppig & Downs 1987). Briefly, ovaries were placed in a 35-mm tissue culture dish containing 2 ml of PBS supplemented with 0.1% collagenase (type CLS I; Worthington Biochemical Corp., Freehold, NJ) and 0.02% deoxyribonuclease I (DNase; Sigma Chemical Co., St.Louis, MO). They were torn apart with forceps and incubated in the enzyme solution with regular agitation by drawing them through a plastic micropipet tip. This procedure yields individual complexes consisting of an oocyte surrounded by one or more layers of granulosa cells. It is thought that the basal lamina that normally surrounds this complex is at least partly degraded by the collagenase treatment. Complexes were washed and then cultured in Eagle's Minimum Essential Medium (MEM) (Invitrogen) supplemented with 1 mg/ml bovine serum albumin (Sigma A4378),0.23 mM pyruvic acid, 50 mg/L streptomycin sulfate and 63 mg/L penicillin G on Costar Transwell-COL membrane inserts (3.0μm, 24mm diameter) in Costar six-well cluster dishes (Costar Corp., Cambridge, MA). Each well contained 2 ml medium below the collagen insert and 2.5ml above the collagen insert. The cultures were incubated at 37°C in 5% CO_2 in air.

Transfection of granulosa-oocyte complexes

Numerous agents – including Lipofectamine2000 (Invitrogen), Metafectene (Biotex, Germany), Hyperfect (Qiagen) and siPORTTM Lipid Transfection Agent (Ambion) – were used to attempt to introduce the siRNAs into the granulosa cells of the GOCs.

Additionally, in some cases, the siRNAs were fluorescently tagged using a commercial kit (Ambion). Transfection reagents were made by mixing 5 μl of the transfection reagent with 250 μl DMEM without serum and incubating at room temperature for 5 min. siRNA at different concentrations were added to 250 μl MEM medium without serum and incubated at room temperature for 5 min. The siRNA and transfection reagents were then combined and incubated at room temperature for 20 min. These transfection mixtures were added to the wells containing the granulosa-oocyte complexes one hour after complex isolation. Forty- eight hours or seventy- two hours after transfection, the oocyte-granulosa cell complexes were dislodged from the membrane by sharply jolting the membrane insert with a snap of a finger against the side of the membrane. The complexes were collected in a 1.5-ml Eppendorf tube and centrifuged at 10000 rpm for 5 min. The the pellet was dissolved in either Trizol reagent (Invitrogen) for total RNA extraction or in 10 μl of 2x SDS sample loading buffer without bromophenol blue for protein quantitation or immunoblot analysis.

Flow cytometry

Twenty- four hours after Cy3-labelled siRNA was transfected into the cultured granulosa-oocyte complexes, trypsin was added to the culture to a final concentration of 0.025% and the suspension was pipetted for 5 min to disaggregate the complexes. The dissagregated cells were collected in a 1.5-ml Eppendorf and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the cell pellet suspended in 1 ml of PBS. The cell pellet was pipetted several times to ensure that single cells were obtained. The samples were placed in a glass tube and flow cytometry was performed using FACScan. Granulosa-

oocyte complexes to which had been added Cy3-labelled siRNA without transfection reagent were used as negative controls.

Culture and transfection of HeLa and NT-1 cells

NT-1 cells were generously supplied by Professor I. Huhtaniemi (Imperial College London, UK). HeLa cells were generously supplied by Professor R. Farookhi (McGill University). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), or DMEM/F12 for the KK-1 and NT-1 cells, supplemented with 10% fetal bovine serum and transfected at 70–90% confluency. For transfection, 4 μg plasmid and 10 μl lipofectamine (Invitrogen) were incubated separately in serum-free medium for 5 min, combined and allowed to stand for 20 min, then added drop-wise to the plate containing the cells. Culture medium was changed 6 h after transfection and siRNA (0 nm, 4 nM, 20 nM, 40 nM) was added. The culture medium was changed 24 hours later. Cells were harvested by scraping or trypsinization 48 h after siRNA transfection. Cells transfected with EGFP plasmid and EGFP siRNA were observed by fluorescence microscopy 48 hours after siRNA addition. NT-1 cells were processed in the same manner except that the plasmid and siRNA were added at a lower cell confluence (30%) and that, 48 hours after siRNA addition, cells were harvested and divided into two aliquots. One was used for RNA extraction and real-time RT-PCR and the other for protein expression analysis.

Real-time RT-PCR

Real-time PCR was used to determine the RNA silencing effect in cultured complexes and NT-1 cells. Total RNA was extracted from cultured complexes and NT-1 cells with

Trizol reagent (Invitrogen). Optical densities at 260 nm and 280 nm were measured to determine the quantity and purity of RNA samples. One μg total RNA was subjected to reverse transcription with MMLV reverse transcriptase (Invitrogen) and random primers (Invitrogen) at 0.5 μg per 20 μl reaction. For real-time PCR, cDNAs were amplified with Qiagene Quantitect SYBR-Green (Qiagen, Mississauga, Ontario) in a Roche Applied Science Lightcycler (Department of Pharmacology and Experimental Therapeutics, McGill University). For each PCR reaction, 0.2 μl of reverse-transcription product was used for 20 μl of final volume. The cycle conditions were as follows: 10 seconds at 94 °C, 10 seconds at 50 °C and 30 seconds at 72 °C for a total of 50 cycles. Melting curve analysis as well as agarose gel electrophoresis were performed on each sample to assess for non-specific amplification and primer-dimer formation.

Primers were designed based on the corresponding published Genbank sequence:

Actin

Forward: 5'-GCTGTGCTATGTTGCTCTAG -3'

Reverse: 5'-ATCGTACTCCTGCTTGCTGA-3';

SSB-1

Forward: 5'-CTGTAGTGAGTGCCGTCTGG-3'

Reverse: 5'-CTGCTGATTTGGATCACTGGT-3'

GAPDH

Forward: 5'-ACCACAGTCCATGCCATCAC-3'

Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'

P450SCC

Forward: 5'-GGAAAGGGAGCTGGTACCTC-3'

Reverse : 5'-TGGGGTCCACGATGTAAACT-3'

KL

Forward: 5'-TCCGAAGAGGCCAGAAACTA-3'

Reverse: 5'-TGCGGCTTTCCTATTACTGC-3'

Immunoblotting

Cultured complexes or NT-1 cells were homogenized in 2x SDS protein sample loading buffer and denatured at 95°C for 5 min. The samples were separated by SDS-Tris-glycine electrophoresis on a 10% polyacrylamide gel. Proteins were transferred onto PVDF membrane (Amersham, Montreal, Quebec) under constant voltage (150 V) for 1 hour. The membrane was subsequently blocked in 5% non-fat milk, in 0.1% Tween-PBS (PBST). The membrane was washed 3 times in PBST and incubated overnight at 4°C with anti-SSB-1 or anti-tubulin antibody in blocking buffer at a concentration of 1/1000. After washing, the membrane was incubated in secondary antibody conjugated to horseradish peroxidase (HRP, Promega, Montreal, Quebec) at a dilution of 1/5000 for 1 hour at room temperature. After the final washes, the fluorescence was revealed using the ECL Plus Kit (Amersham). The results were quantitated with a phosphoimager (Amersham Biosciences, Montreal, QC, Canada).

Radioimmunoassay measurement of progesterone production

NT-1 cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum on a 10-cm diameter plate. When the cells reached 30% confluency, they were

harvested and counted, and $2x10^5$ cells per well were added to 6-well plates in a volume of 2 ml of medium. Twenty-four hours later, when the cells had reached ~30% confluency, the siRNA and transfection reagent mixture were added. Following 40-hour incubation, medium was changed and 8 hours later, 1 ml of medium was removed and stored at -80°C for the progesterone measurement using RIA methods. Therefore, the assay measured the amount of progesterone secreted by the cells in 8 hours.

Liquid-phase RIA was used to determine the progesterone concentrations. Progesterone-l la-glucuronide-' 2 5 I-iodotyramine (Amersham) was used as radioactive tracer, and goat antirabbit IgG (Prince Laboratories, Toronto, ON, Canada) as the precipitating second antibody. The sensitivity of the assay was 6 pg/ml and the intra- and inter-assay coefficients of variation were less than 13%.

Results

Both *EGFP* siRNA and *SSB-1* siRNA efficiently decrease target protein expression in immortalized cell lines.

SSB-1 protein is expressed in ovarian granulosa cells (Xing et al, 2006). To test its function, we attempted to employ RNAi-based methods to reduce its quantity in these cells. The most effective method for RNAi-based silencing in somatic cells employs chemical synthesized short interfering RNAs (siRNA). However, since the rules governing the interaction of the siRNAs with the target mRNA and its subsequent translational silencing or degradation are not completely understood, it is typically necessary to test several sequences to find effective ones. To establish the siRNA technique, we first sought to silence a transfected product. Plasmid encoding full length EGFP was transfected into HeLa cells. Six hours after transfection, siRNA was added to the culture medium or no addition was made. 48 hour after siRNA addition, *EGFP* expression was assessed by fluorescence microscopy (Figure 1). As compared to the transfected controls that did not receive siRNA, EGFP fluorescence was reduced by siRNA ranging from 4 nM to 40 nM. These results established that the *EGFP* siRNA efficiently silenced *EGFP* expression.

Next, we tested the ability of siRNA to silence expression of *SSB-1*. HeLa cells were transfected with a construct encoding SSB-1 (Xing et al, 2006). Six hours after transfection, siRNA corresponding to *SSB-1* or to *EGFP* was added to the medium. Forty-eight hours after siRNA addition, cells were harvested and analyzed by immunnoblotting. Three concentrations of *SSB-1* siRNA #83484 (4 nM, 20 nM, 40 nM) each decreased the

SSB-1 expression level more then 90% (Figure 2). Only the 31 kDa band was quantitated as it was strong and stable compared to the 36 kDa band. *EGFP* siRNA appeared to slightly decrease *SSB-1* expression as compared to controls that did not receive siRNA. The experiment was performed twice and similar results were obtained each time. We conclude that siRNA #83484 can effectively decrease SSB-1 protein expression.

SSB-1 siRNA does not efficiently reduce _SSB-1_ mRNA expression in granulosa-oocyte complexes

Most transfection techniques appear to be considerably more effective using immortalized cells as compared to primary cells. To examine whether siRNAs could be efficiently introduced into the granulosa cells of GOCs, the *SSB-1* siRNA was labelled with Cy3 and labelled siRNA was added into cultured GOCs together with Lipofectamine2000 (Invitrogen), Metafectene (Biotex, Germany) or Hyperfect (Qiagene). 48 hours after transfection, the granulosa-oocyte complexes were transferred onto glass slides and observed by fluorescence microscopy.

We found that many of granulosa-oocyte complexes subjected to transfection with Cy3-labelled siRNA showed bright fluorescence (Figure 3A). It may be noted that fluorescence was restricted to the granulosa cells (Figure 3A, c and d), implying that the siRNA did not enter the oocytes in detectable quantities. Fluorescence was barely detectable when Cy3-labelled siRNA was added in the absence of lipofectamine2000 (Figure 3B). We tested other transfection reagents, but observed no major reproducible differences among those tested and they all worked (Figure 3C).

We attempted to obtain a preliminary estimate of the efficiency of transfection – that is, the percentage of cells that took up the siRNA. To this end, granulosa-oocyte complexes were transfected as above and 24 hr later were disaggregated into a single-cell suspension. Cells were separated into fluorescent and non-fluorescent groups by flow cytometry. The results are shown in Figure 4. As compared to negative control group (no lipofectamine added to the cultured complexes) shown in Figure 4A, the transfected cells group showed at least 90% fluorescence shift (Figure 4B). These results indicate that the Cy3-labelled siRNA was present in a high percentage of the cells, implying a high transfection efficiency.

We then tested whether levels of *SSB-1* mRNA were reduced. Granulosa-oocyte complexes were isolated and transfected as described above. Forty-eight hours later, cells were recovered and *SSB-1* mRNA was assayed using real-time RT-PCR. SiRNA concentrations of 20nM, 40nM, 100nM were able to slightly reduce *SSB-1* mRNA expression level. However, it remained at least 70% of control (Figure 5). SiRNA at 200 nM caused all complexes to degenerate within 48 hr after transfection. This suggested that *SSB-1* mRNA was resistant to attack by siRNA.

To understand the reason for the inefficient silencing, we transfected the granulosa-oocyte complexes using a commercially available siRNA targeting *GAPDH*. This siRNA decreases *GAPDH* RNA levels by at least 70% in several cell lines. In contrast to these results, we were unable to achieve a detectable decrease in *GAPDH* RNA expression in

the granulosa-oocyte complexes (data not shown). These data indicate that, at least under the experimental conditions employed here, siRNA may not be an effective tool to selectively deplete specific mRNA populations in granulosa-oocyte complexes.

siRNA reduces expression of endogenous *SSB-1* in an immortalized murine granulosa cell line

As we were unable to reduce *SSB-1* expression in primary granulosa cells within granulosa-oocyte complexes, we next tested an immortalized cell line, NT-1, that was derived from granulosa cell tumours of transgenic mice expressing SV40 T-antigen under the control of the inhibin α-subunit promoter (Rilianawati et al., 1999). The cells were incubated with the siRNA combined with transfection reagent, transferred to fresh medium and harvested 48 hours later. mRNA levels was quantitated by real-time PCR Two different siRNAs targeting SSB-1 were used as well as an siRNA targeting EGFP as control.

As shown in Figure 6A, the SSB-1 siRNAs both individually and in combination reduced *SSB-1* mRNA by approximately 80%, as compared to the amount present in cells transfected with the EGFP siRNA. In contrast, all groups contained similar quantities of mRNA encoding *GAPDH*. This implies that the SSB-1 siRNA specifically reduced *SSB-1* mRNA quantity. To further characterize the effect of the siRNA, cells were transfected and harvested for analysis at 48 hours, 72 hours and 120 hours (5 days) after transfection. As shown in Figure 6B, SSB-1 mRNA was lowest 48 hours after transfection and then

began to increase. This indicates that, as expected, the inhibitory effect of the siRNA is transient.

We previously showed that anti-SSB-1 recognizes proteins migrating at 31 kDa, 36 kDa and 40 kDa in NT-1 cells, which is the same pattern observed in whole ovaries and isolated ovarian follicles (Xing et al., 2006). To test whether the loss of *SSB-1* mRNA was accompanied by a decrease in SSB-1 protein, we first tested whether the siRNA could prevent the expression of *SSB-1* introduced by transfection. Plasmid encoding SSB-1 was transfected into the NT-1 cells together with siRNA at different concentrations. Forty-eight hours after transfection, the cells were harvested and SSB-1 was assayed by immunoblotting. In cells transfected with the SSB-1 plasmid but no RNAi, the 31 kDa immunoreactive species became more prominent (Figure 7A). In contrast, this increase was attenuated or failed to occur in cells co-transfected with RNAi. These results indicated that the siRNA targeting *SSB-1* could prevent expression of *SSB-1* introduced by transfection.

We then tested whether expression of endogenous SSB-1 could be reduced in non-transfected NT-1 cells. Cells were transfected with siRNA targeting SSB-1 or EGFP or were mock-transfected. Forty-eight hours later, the cells were harvested and protein levels assayed by immunoblotting. As shown in Figure 7B, both the 31 kDa and 36 kDa species were substantially reduced in the cells transfected with the SSB-1 siRNA. To verify this result, cells were harvested at 48, 72, and 120 hours after transfection. As shown in Figure 7C, SSB-1 protein declined and then began to increase. This pattern is similar to

that observed for the encoding mRNA. In contrast, SSB-1 protein remained relatively constant in cells transfected with EGFP siRNA.

Loss of SSB-1 impairs granulosa cell gene expression and function

To test whether the reduction in SSB-1 affected the function of the granulosa cells, we first tested expression of two genes that are expressed in granulosa cells and serve defined functions. P450scc, the cholesterol side-chain cleavage enzyme, catalyzes the first step in synthesis of all steroid hormones; namely, the conversion of cholesterol to pregnenolone. Its encoding gene is termed *cyp11a* (Keeney *et al.* 1995). We found that, in NT-1 cells analyzed forty-eight hours after transfection with siRNA targeting *SSB-1*, the quantity of mRNA encoding P450scc was decreased by about 40% and the result is significant (Figure 6A, part c). Moreover, as the amount of *SSB-1* mRNA increased with prolonged incubation, the amount of *cyp11a* mRNA (encoding P450scc) also increased (Figure 6B, part b). Interestingly, expression of Kit ligand (KL), which is secreted by granulosa cells and interacts with the c-Kit receptor on the oocyte surface to stimulate oocyte growth (Klinger & De Felice 2002), was decreased around 30% in SSB-1depleted NT-1 cells and the result is significant.

To test whether the decrease in mRNA encoding P450scc led to a decrease in the protein, NT-1 cells were transfected and cultured for 48 hours as above, then assayed by immunoblotting. As shown in Figure 8, P450scc protein declined by about 50% in the cells treated with *SSB-1* siRNA as compared to those treated with *EGFP* siRNA. In contrast, the quantity of $p42^{Erk2}$ and $p44^{Erk1}$, as well as of two unknown proteins

recognized by the anti-P450scc antibody, remained unchanged. These results indicate that depletion of SSB-1 using siRNA in NT-1 cells caused a depletion of P450scc.

Because P450scc catalyzes the first step in steroidogenesis, we carried out preliminary experiments to test whether this process was affected in the SSB-1-depleted NT-1 cells. To this end, we measured progesterone production, as this is secreted by many immortalized granulosa cell lines including NT-1 cells (Rilianawati *et al.* 1999). RNAi was performed as above and the cells were transferred to fresh medium 40 hours later. After an 8-hour incubation, the supernatants were collected to measure the progesterone production by radioimmunoassay and the cells were harvested to measure the amount of SSB-1 and P450scc by quantitative RT-PCR and immunoblotting. The RNA and protein levels change of SSB-1 and P450scc after RNAi silencing were quite stable in several groups of experiments. We observed that progesterone production was reduced by about 40% in cells transfected with either SSB-1 siRNA as compared to those transfected with EGFP siRNA group (Figure 9). However, the total amount of progesterone measured in this experiment was relatively low compare to published result (Rilianawati *et al.* 1999). Therefore, these results are consistent with the possibility, but do not establish, that the normal quantity of SSB-1 protein is required for NT-1 cells to maintain progesterone production.

Discussion

We have attempted to use RNAi-base methods to reduce the quantity of SSB-1 in granulosa cells. We focused initially on freshly isolated granulosa cells that were associated with oocytes in granulosa-oocyte complexes. The primary granulosa cells appeared to take up fluorescently-labelled siRNAs, as judged by examination using fluorescence microscopy and by FACS sorting. We were unable, however, to achieve substantial reduction in the level of the targeted RNAs. At least two explanations may be offered for this result. First, the siRNAs may not have been delivered to the appropriate compartment of the cells. Liposome-based transfection agents usually enter the cell by endocytosis (rarely, direct fusion with the plasma membrane) after which the endosome must be destroyed to release the contents into the cytoplasm (Zhdanov *et al.* 2002). It is possible that this release did not occur efficiently. Alternatively, the siRNA may not trigger degradation of the target mRNA in these primary cells. However, as no effect was seen using siRNA targeting GAPDH, which is known to lead to loss of target mRNA in different cell lines, this would imply that siRNA operates differently in primary and immortalized cells.

Other methods exist that may permit siRNA delivery into granulosa cells. We attempted to introduce fluorescent siRNA by electroporation, but no fluorescence was observed using conditions that permitted cell survival (data not shown). It is also possible to express the siRNA from DNA under the control of a strong promoter, such as the RNA-polymerase III dependent promoter (H1-RNA promoter) (bbas-Terki *et al.* 2002). Adenovirus-based vectors efficiently infect many cell types and integration into the

genome does not requie DNA replication (Rinne *et al.* 2006). Therefore, this method might allow expression of siRNA in transfected cells. Over the last several years, however, cell-penetrating peptides (CPP) have attracted much interest. These are short (about 10 amino acids) peptides that are typically rich in arginine and efficiently cross the plasma membrane. Two CPPs that are particularly well-known are derived from the HIV Tat protein and from the Antennapedia protein of *Drosophila* (Futaki 2006). Various molecules may be linked to CPPs, thus providing a means to introduce them into the cytoplasm. As siRNA presumably exerts its activity in the cytoplasm, rather than the nucleus, CPPs could be an effective delivery system.

Using the immortalized NT-1 granulosa cell line, we were able to deplete both the encoding mRNA and SSB-1 protein. Both had declined substantially by 48 hr after delivery of the siRNA. Moreover, both increased over the next few days, and the kinetics were similar within the limitations of the analysis. This suggests that SSB-1 protein in NT-1 cells is relatively rapidly turned over – hence its decline paralleled the loss of mRNA – and that the *SSB-1* gene is constitutively transcribed – hence the recovery of the mRNA several days after siRNA delivery.

Cells lacking SSB-1 also showed decreases in mRNA encoding P450scc and Kit ligand. In contrast, the amount of mRNA encoding GAPDH remained unchanged. Moreover, the amount of mRNA encoding P450scc subsequently decreased in parallel with the decrease in SSB-1 mRNA. Consistent with the established role for P450scc in steroid synthesis, preliminary data indicated that progesterone production was decreased in cells lacking

SSB-1. Taken together, these results suggest that SSB-1 is required to maintain progesterone production in NT-1 cells and that this is mediated through an effect on the amount of mRNA encoding P450scc.

The mechanistic link between SSB-1 and P450scc mRNA remains to be identified. SSB proteins have been shown to interact with the MET tyrosine kinase receptor through their SPRY domain. Moreover, co-expression of SSB-1 increased the transcription of a reporter gene in response to HGF, the ligand for MET(Wang *et al.* 2005). Thus, perhaps by increasing the activity of ligand-bound MET, SSB-1 is able to up-regulate the transcription of certain genes. MET expression has been reported in primary granulosa cells (Parrott & Skinner 1998; Ito *et al.* 2001; Zachow *et al.* 2000) and cell lines (Taniguchi *et al.* 2004) and both primary granulosa cells and cell lines are responsive to HGF (Ito *et al.* 2001; Taniguchi *et al.* 2004) implying that they express functional MET. Thus, SSB-1 could augment MET-dependent activity in granulosa cells.

Activated MET has many targets, among these is c-Jun terminal kinase (JNK), which phosphorylates and thereby activates the transcription factor, c-Jun (Royal *et al.* 2000). Interestingly, c-Jun is able to function synergistically with steroidogenic factor (SF)-1 to increase transcription from the *Cyp11a1* (P450scc) promoter in steroidogenic cells but not in non-steroidogenic cells (Huang *et al.* 2001). These observations are consistent with the possibility that activated MET could, via phosphorylation of c-Jun, increase transcription of *Cyp11a1*, and that SSB-1 could augment this MET-dependent response. Moreover, as granulosa cells undergo a differentiation process termed luteinization following ovulation,

it will be important to examine whether the potential link between SSB-1, MET activation, and steroid synthesis is altered following luteinization.

The mechanistic link between SSB-1 and KL mRNA remains to be identified. SSB-1 can increase the transcriptional activity induced by HGF and MET binding (Wang *et al.* 2005). HGF is growth factor produced by the thecal cells, and KL is expressed in granulosa cells. MET is expressed in thecal and granulosa cells. It has been shown that there is a feed back loop between thecal cells and granulosa cells that is mediated by HGF and KL. HGF can stimulate the KL expression in granulosa cells and KL can stimulate the HGF expression in thecal cells (Parrott *et al.*1998). SSB-1 might take part in this loop through an interaction with MET.

Acknowledgements

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Figures

Figure 1

siRNA-mediated silencing of expression of transfected EGFP.

HeLa cells were transfected with a plasmid encoding EGFP. Six hr later, siRNA was added to a final concentration 0, 4, 20 or 40 nM. Each condition was replicated three times. 48 hours after siRNA addition, cells were observed by fluorescence microscopy using the filter set for FITC-type fluorochromes. Cells that did not receive siRNA show bright fluorescence, whereas the cells that received siRNA show very weak or no fluorescence.

(A) EGFP plasmid only, no siRNA. (B) EGFP plasmid and 4 nM EGFP siRNA. (C) EGFP plasmid and 20 nM EGPF siRNA. (D) EGFP plasmid and 40 nM EGPF siRNA. Magnification A,B x100, C,D x400.

Figure 1

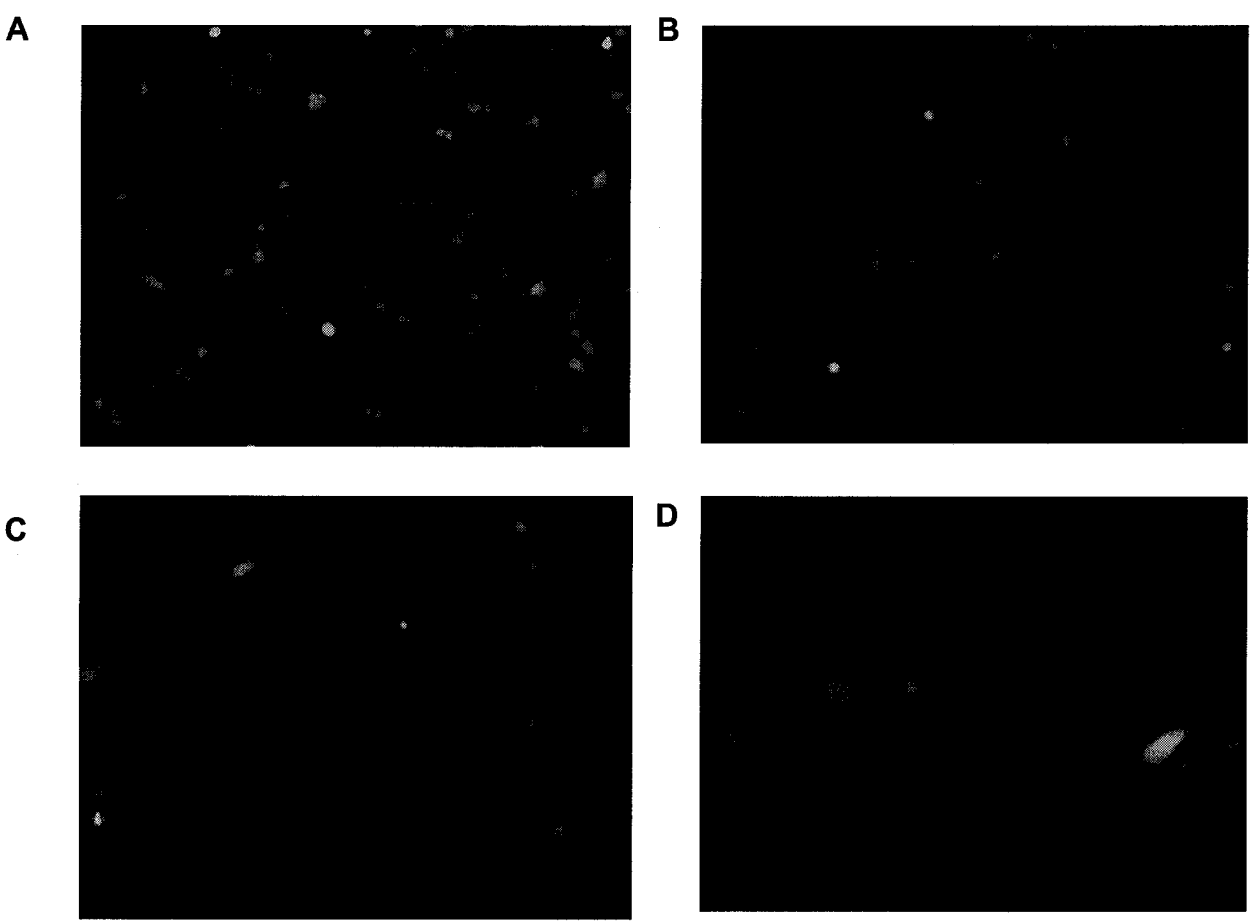
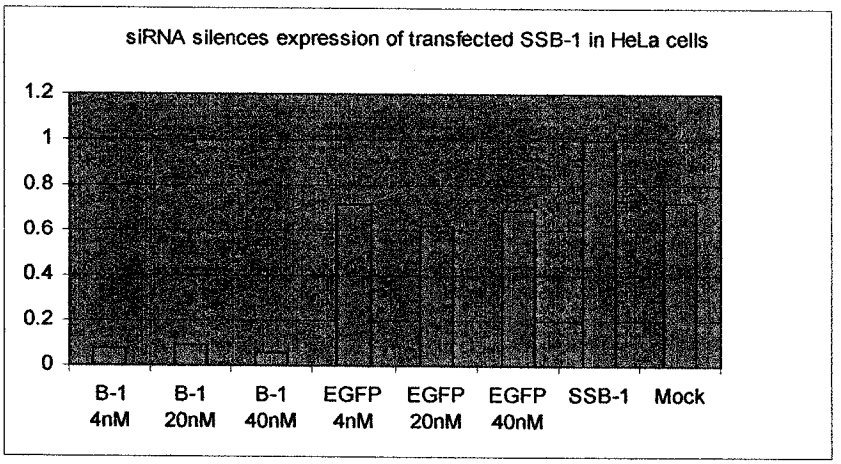


Figure 2

siRNA-mediated silencing of expression of transfected SSB-1.

HeLa cells were transfected with a plasmid encoding SSB-1. Six hr later, siRNA targeting either SSB-1 or EGFP was added to a final concentration 4, 20 or 40 nM (in a 6-well plate, the amount of siRNA added to each well is 0, 10, 50, 100 pmol). Three replicates were performed for each condition. 48 hours after siRNA addition, cells were harvested and processed for immunoblotting using anti-SSB-1 and anti-tubulin antibodies. Signals were quantified using a phosphorimager. The figure shows a representative blot. Upper panel: Quantification of SSB-1/tubulin immunoblotting signals of one experiment. SSB-1 siRNA decreases SSB-1 protein expression by ~90%. Middle panel: Immunoblot using anti-SSB-1. Lower panel: Immunoblot using anti-tubulin. Samples are in the same order in all three panels.

Figure 2


siRNA silences expression of transfected SSB-1 in HeLa cells
1.2
1
0.8
0.6
0.4
0.2
0
B-1 4nM
B-1 20nM
B-1 40nM
EGFP 4nM
EGFP 20nM
EGFP 40nM
SSB-1
Mock

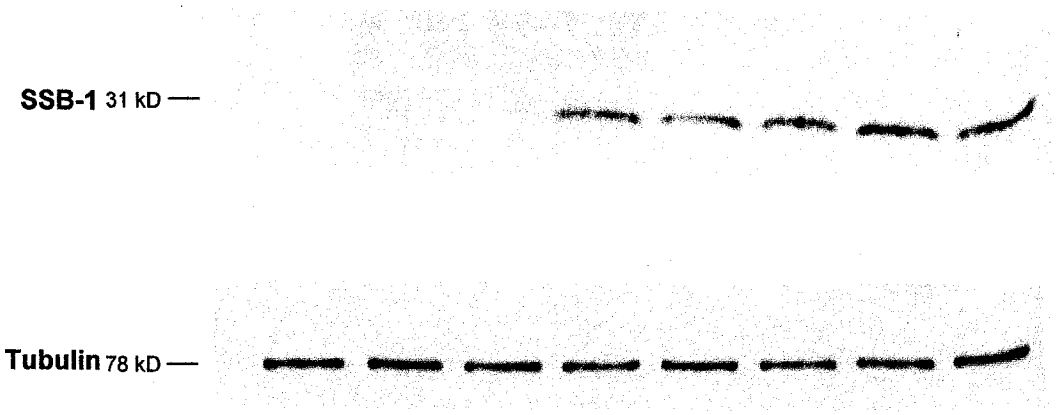

SSB-1 31 kD
Tubulin 78 kD

Figure 3

Cy3 labelled siRNA can be transfected into granulosa-oocyte complexes.

(A) Cy3-labelled siRNA was transfected into the cultured granulosa-oocyte complexes using lipofectamine2000.

a, b: EGFP siRNA, 43h after transfection, Scale bar: 100 μm.

c, d: SSB-1 siRNA, 43h after transfection, Scale bar: 50 μm.

a, c: under bright field microscope, b, d: under fluorescent microscope.

(B) Cy3-labelled siRNA was transfected into granulosa-oocyte complexes using lipofectamine2000.

a, c: EGFP siRNA added to the cultured complexes together with lipofectamine 2000 and detected 43 hours after transfection. Scale bar: 100 μm.

b, d: EGFP siRNA was added to the cultured complexes without lipofectamine 2000 and detected 43 hours after transfection. Scale bar: 100 μm.

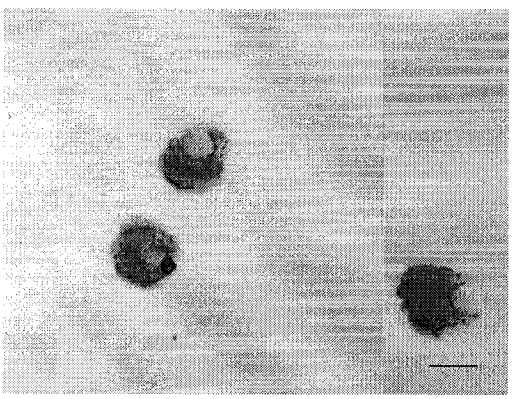
a, b: under fluorescent microscope. c, d: under brightfield microscope.

(C) Cy3-labelled siRNA was transfected into granulosa-oocyte complexes using different reagents. (a) Metafectene (b) Lipofectamine2000 (c) Hyperfectene. No consistent differences were observed. Scale bar: 20μm.

Figure 3

A

a



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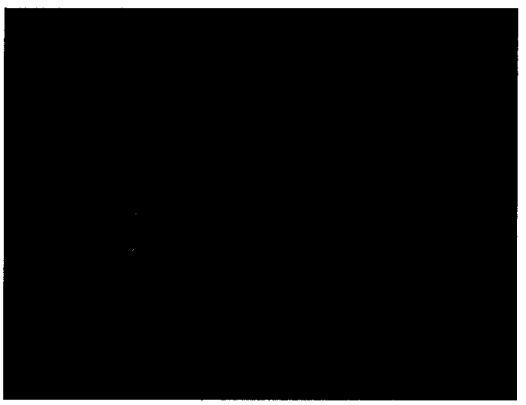

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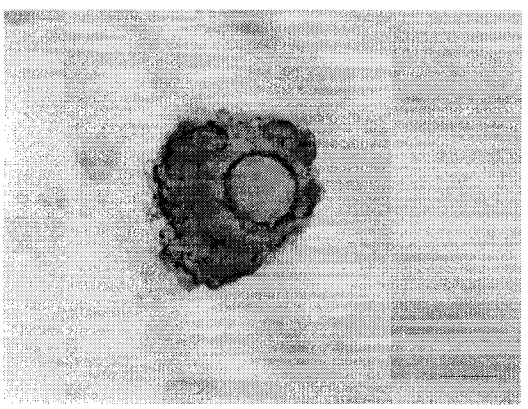

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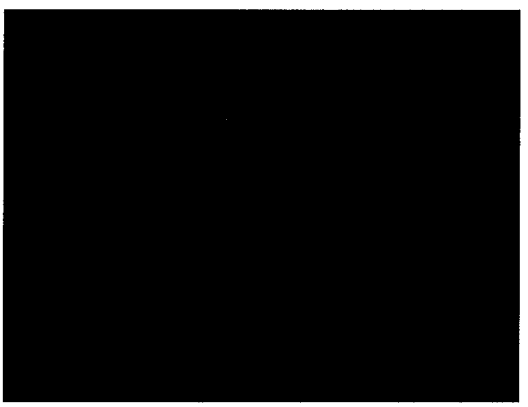


Figure 3

B

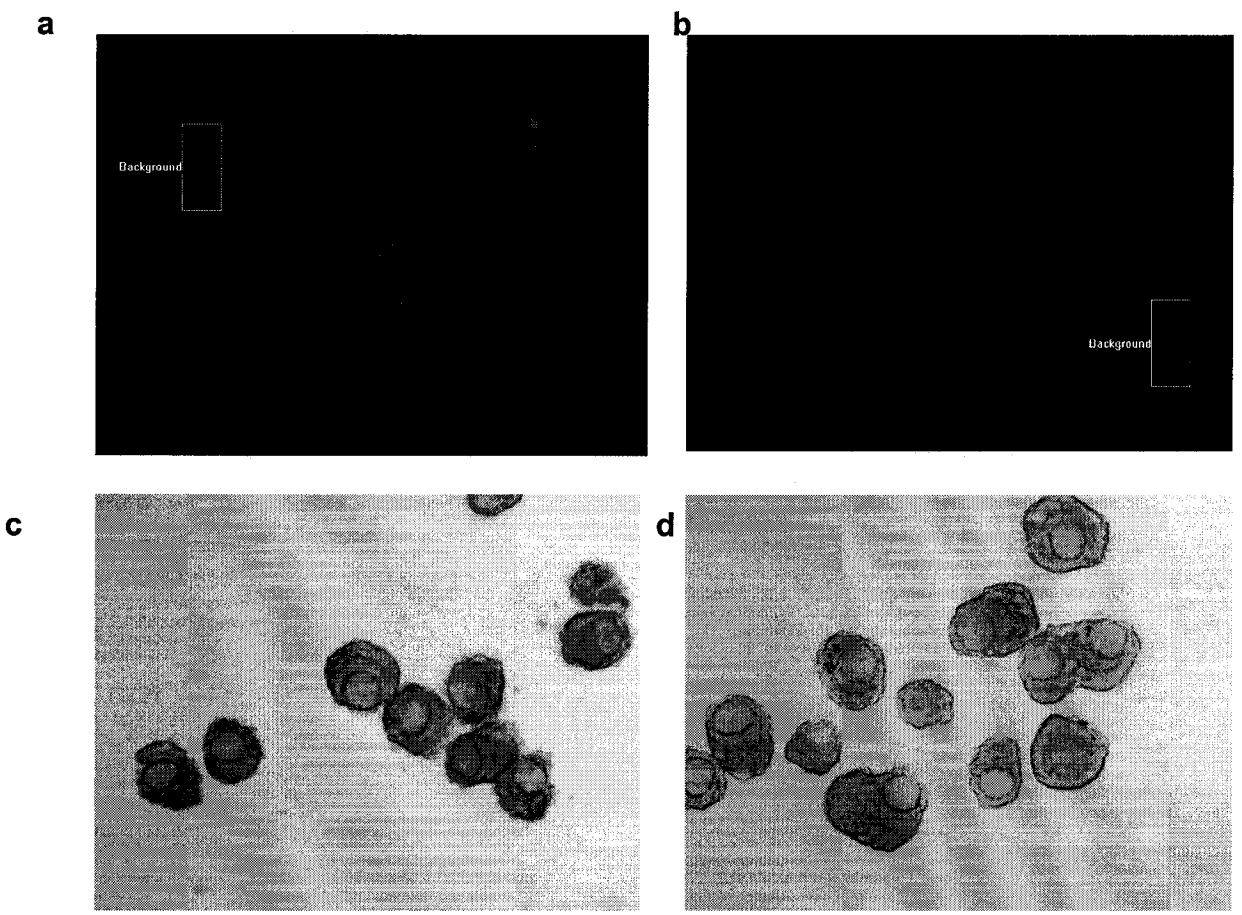
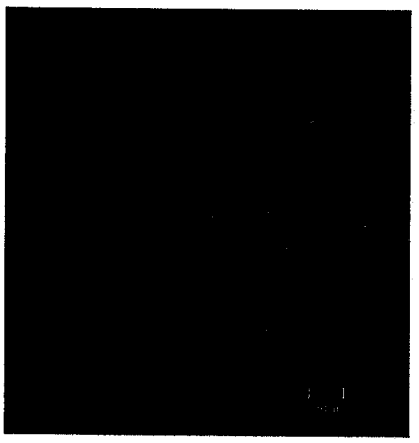


Figure 3

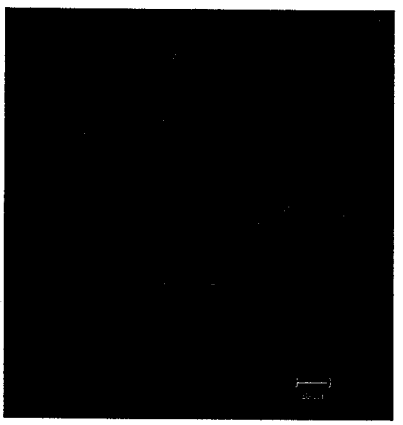
C

a



Metafectene

b



Lipofectamine2000

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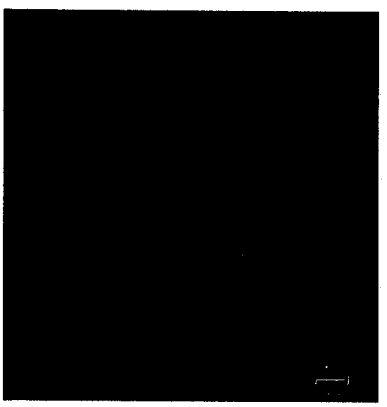


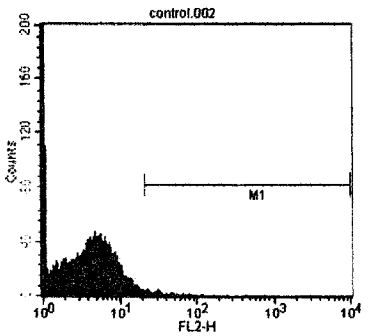
Hyperfectene

Figure 4

Efficiency of transfection as verified by flow cytometry. Granulosa-oocyte complexes were combined with Cy3-labelled siRNA in the absence (A) or presence (B) of lipofectamine2000. Twenty-four hours later, they were disaggregated into single-cell suspensions and fluorescence was analyzed by flow cytometry. The extensive shift in fluorescence in (B) as compared to (A) indcates that at least 90% transfection efficency was obtained.

Figure 4

A



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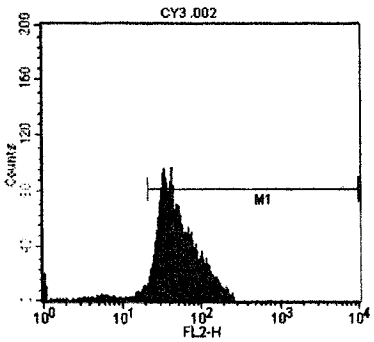
Gated Events: 7890
X Parameter: FL2-H (Log)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	7890	100.00	78.90	5.41	3.67	136.14	3.86	1
M1	21, 9910	162	2.05	1.62	41.21	36.37	65.86	32.20	22

B



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Acquisition Date: 16-Nov-05
Gated Events: 9623
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Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	9623	100.00	96.23	53.63	44.17	65.11	42.55	39
M1	21, 9910	9028	93.82	90.28	56.39	49.10	60.74	44.51	39

Figure 5

siRNA targeting SSB-1 only modestly reduces the mRNA quantity following transfection of granulosa-oocyte complexes. Complexes were subjected to the standard transfection procedure. *SSB-1* mRNA was assayed using real-time RT-PCR. The data shown are the combined results of 6 trials using lipofectamine2000, 10 trials using metafectene, and 3 trials using hyperfectene.

Figure 6

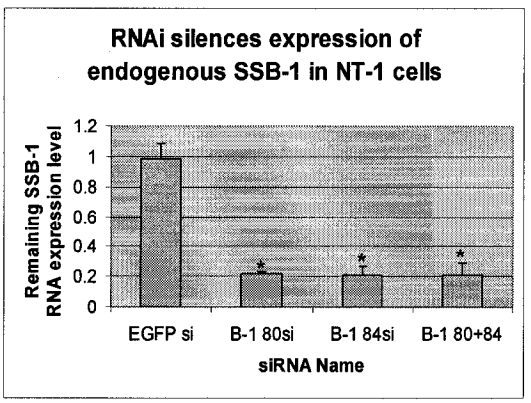
B-1 siRNA reduces selected mRNAs in NT-1 cells. (A) Cells were transfected with EGFP siRNA or SSB-1 siRNA (100 pmol of #80, 100 pmol of #84, 50 pmol #80 and 50 pmol#84). 48 hours after transfection, cells were harvested and quantitative RT-PCR was performed using primers specific to SSB-1, GAPDH, P450scc, KL. Results were normalized to actin used as an endogenous control. The mean and variation of three independent experiments, each done in triplicate, is represented. Paired student's t-test was used to compare each group of B-1 siRNA sample to EGFP siRNA sample. $p<0.05$ for SSB-1, P450SCC, KL and $p>0.05$ for GAPDH.

B. SSB-1 and P450scc RNA increase beyond 48 hr after B-1 siRNA transfection. Cells were transfected using EGFP siRNA and SSB-1 siRNA and harvested and analyzed 2day, 3day and 5 days later. Real-time RT-PCR was used to quantitate the SSB-1 and P450scc levels, using actin as endogenous control. The mean and variation of three independent experiments, each done in triplicate, is represented. Three group of B-1 siRNAs all reduced *SSB-1* mRNA by approximately 80%, reduced P450scc RNA expression by 40% and KL expression by 30%, as compared to the amount present in cells transfected with the EGFP siRNA. All groups contained similar quantities of mRNA encoding *GAPDH*. Paired student's t-test is used to compare each group of B-1 siRNA sample to EGFP siRNA sample. $p<0.05$ for all experiments.

Figure 6

A

a



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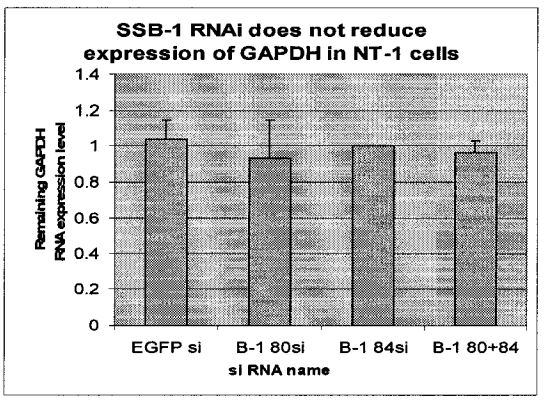

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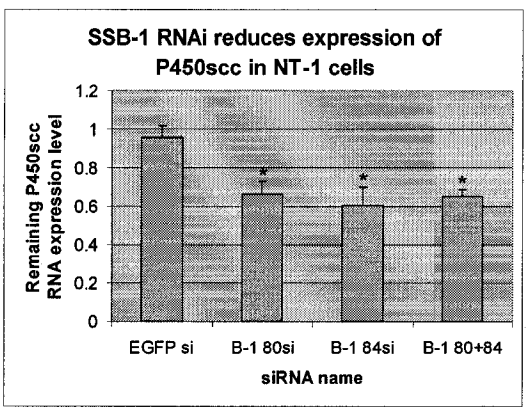

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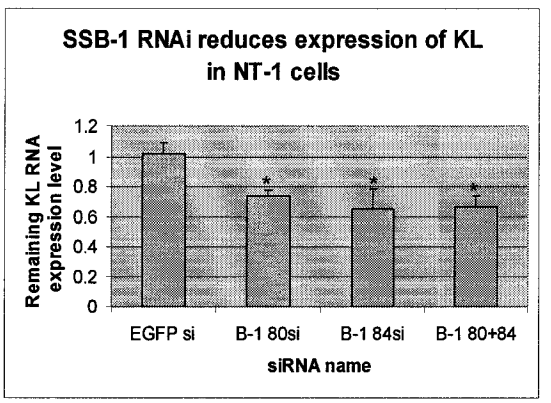


Figure 7

SSB-1 siRNA can silence SSB-1 protein expression in NT-1 cells.

(A) siRNA can silence transfected SSB-1. Plasmid containing the coding region of SSB-1 was transfected into NT-1 cell together with different concentrations of B-1 siRNA. Cells were harvested and analyzed by immunoblotting 48 hr later. (B) SSB-1 siRNA can reduce endogenous SSB-1 protein. Cells were mock-transfected (transfection reagent alone) or transfected with EGFP siRNA or SSB-1 siRNA, then harvested and analyzed by immunoblotting 48 hr later. Three independent experiments were performed. A representative experiment is shown. Upper panel: Phosphorimager data. Middle panel: Immunoblot using anti-SSB-1. Lower panel: Immunoblot using anti-tubulin. (C) SSB-1 protein increases beyond 48 hr after B-1 siRNA transfection. Cells were transfected using EGFP siRNA and SSB-1 siRNA and harvested and analyzed 48 hr, 72 hr and 5 days later. Three independent experiments were performed with EGFP siRNA and SSB-1 siRNA. Immunblots were quantified using a phosphoimager, using tubulin as endogenous control. The experiment was performed three times. Paired student's t-test was used to compare each group. $p<0.05$ for 2day and 3day and $p>0.05$ for 5 day experiment.

Figure 7

A

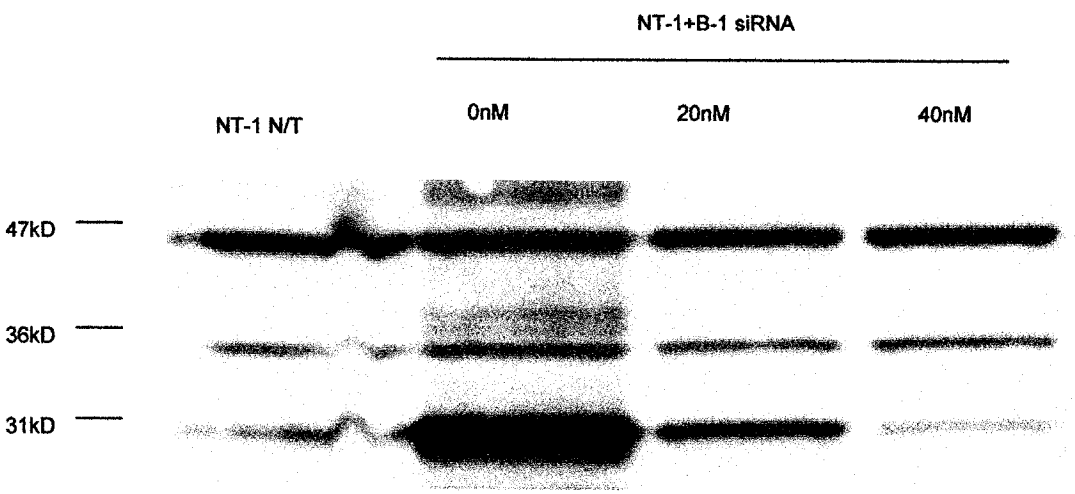
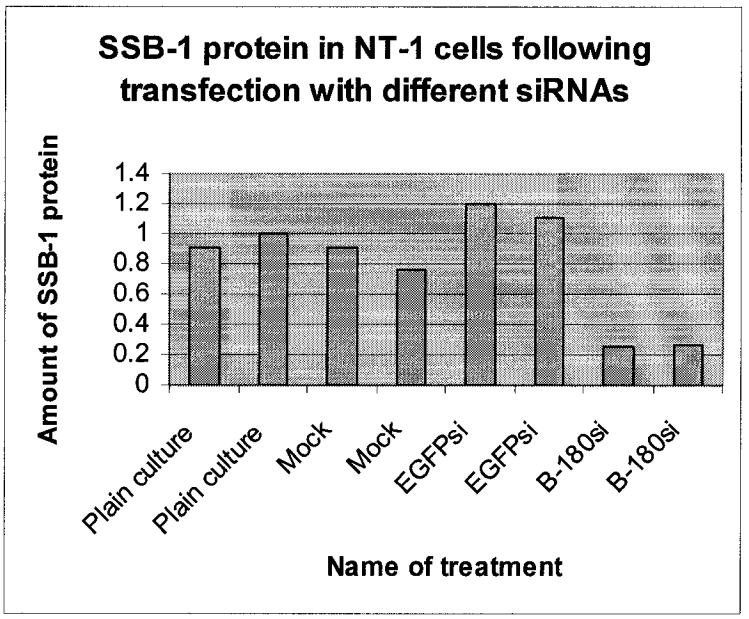


Figure 7

B


SSB-1 protein in NT-1 cells following transfection with different siRNAs
Amount of SSB-1 protein
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Plain culture
Plain culture
Mock
Mock
EGFPsi
EGFPsi
B-180si
B-180si
Name of treatment

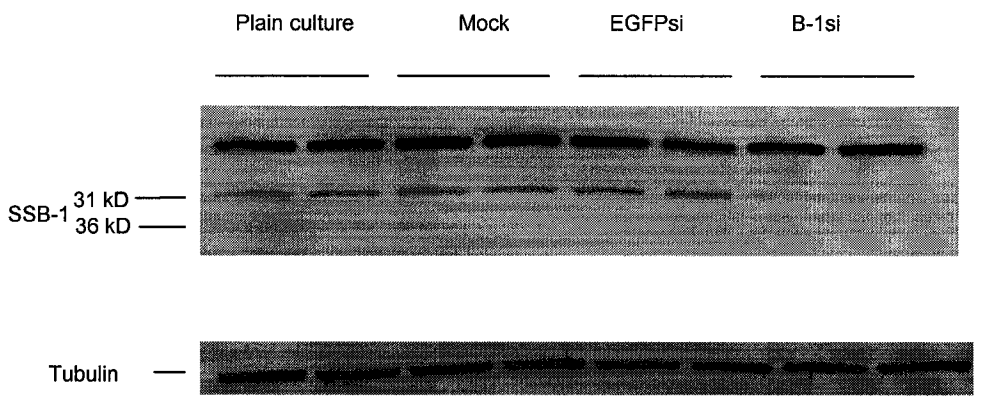

Plain culture
Mock
EGFPsi
B-1si
SSB-1
31 kD
36 kD
Tubulin

Figure 7

C

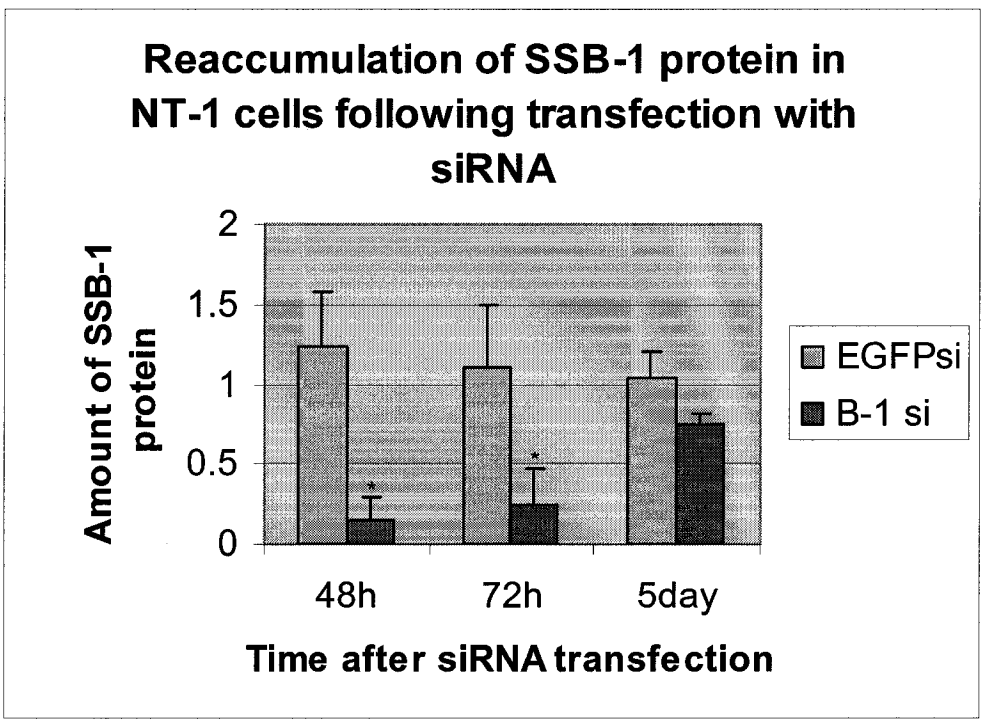
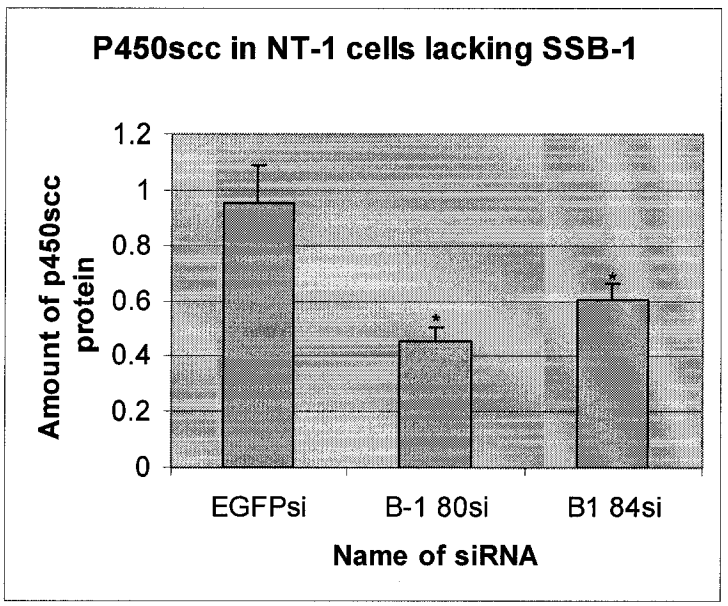

Reaccumulation of SSB-1 protein in NT-1 cells following transfection with siRNA
Amount of SSB-1 protein
2
1.5
1
0.5
0
48h
72h
5day
Time after siRNA transfection
EGFPsi
B-1 si

Figure 8

Effect of SSB-1 RNAi on P450scc protein. Cells were transfected using EGFP siRNA and SSB-1 siRNA and harvested and analyzed 48 hr later. Upper panel: Phosphorimager data. Middle panel: Immunoblot using anti-P450scc. Lower panel: Immunoblot using anti-ERK. Three independent experiments were performed. Both B-1 siRNA group reduced P450scc protein expression to 60%. Paired student's t-test was used to compare EGFPsi sample to SSB-1si sample, $p<0.05$.

Figure 8


P450scc in NT-1 cells lacking SSB-1
Amount of p450scc protein
1.2
1
0.8
0.6
0.4
0.2
0
EGFPsi
B-1 80si
B1 84si
Name of siRNA

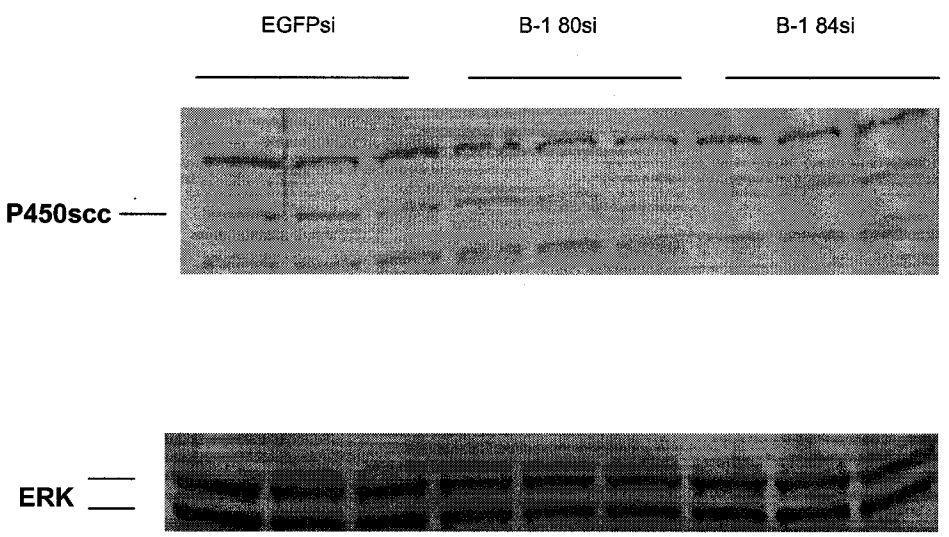
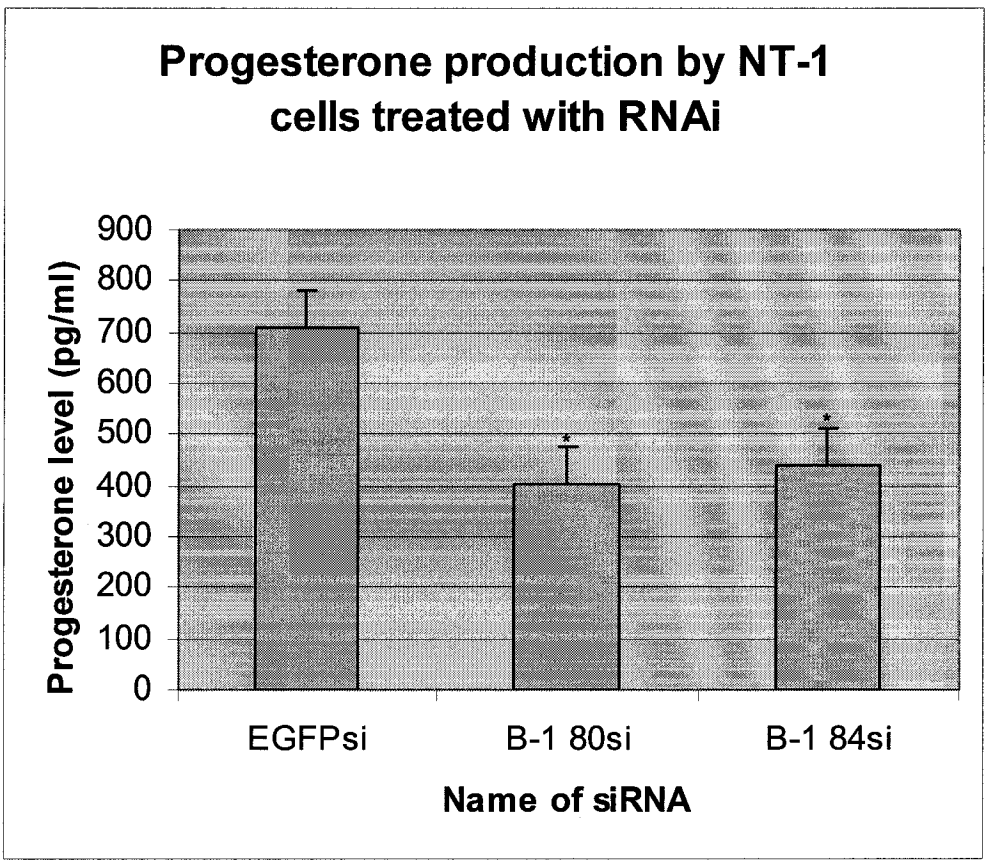

EGFPsi
B-1 80si
B-1 84si
P450scc
ERK

Figure 9

Effect of SSB-1 RNAi on progesterone production by NT-1 cells. NT-1 cells were transfected using EGFP siRNA or SSB-1 siRNA. Forty hr later, they were transferred to fresh medium. After a further 8 hr of culture, medium was collected and progesterone production was measured by radioimmunoassay. Results show the mean of six replicates. Paired student's t-test was used to compare EGFPsi sample to SSB-1si sample, $p<0.05$.

Figure 9


Progesterone production by NT-1 cells treated with RNAi
Progesterone level (pg/ml)
900
800
700
600
500
400
300
200
100
0
*
*
EGFPsi
B-1 80si
B-1 84si
Name of siRNA

5. APPENDIX I

Identified murine homologue of Drosophila Gustavus SSB-1 with Race

Connecting text

When I began working on this project, I did not have sequence information of murine homologues of *Drosophila gus*. I obtained a 300-bp expressed sequence tag (EST) through searching the NCBI gene bank for mouse sequences that were similar to *gus*. Using the technique termed **R**apid **A**mplification of **C**omplementary cDNA **E**nds (RACE), I obtained the full-length cDNA sequence containing this EST region from mouse ovary total RNA. This sequence was later posted in GenBank and named SSB-4 by another group. I did not work on SSB-1 until the sequence had been posted by the other group. I then found SSB-1 to have very high sequence similarity to SSB-4 and to be expressed more abundantly than SSB-4 in ovary and testis tissues. Therefore, I switched my concentration to SSB-1 for my later expression and functional research. This appendix describes the RACE work.

Introduction

To initiate this project, the NCBI GenBank was searched for mouse sequences that were similar to the *Gus* gene of *Drosophila* (NP_724402). This search yielded a 300-bp expressed sequence tag (EST). I first tested whether this EST was expressed in ovary and testis tissue with RT-PCR. After I confirmed the expression, I decided to obtain the full length cDNA using the technique named **R**apid **A**mplification of **C**omplementary cDNA **E**nds (Race). Thermal RACE (also known as single-sided or anchored PCR) is a technique through which previously unobtained 3' and 5' ends of cDNA can be amplified starting with the knowledge of a small region of internal cDNA (in this project, it is a short region of the Gus-related EST). This technique provides an alternative to construct and screen conventional libraries (Frohman 1993). The rationale of this method is to use gene-specific primers in the region of EST and a PCR anchor primer attached to the poly(A) tail of the 3' end, or synthetic 5' end polyA tail, through series sequencing, to get the unobtained region attached to the EST (Figure 1).

This method yielded two candidate murine homologues of *Drosophila Gus*, which were termed *Gus1* and *Gus2*. Half a year after I got these sequences, the *Gus1* sequence was posted on the NCBI website (AAL57358, 14-Aug-2002) and was termed *SSB-4*. *Gus2* was published on the website more recently (BAC38995, 02-Sep-2005) as an unnamed protein product. I then named it *similar to SSB-4*.

SSB-1 was first posted on the NCBI website (AAL57355, 02-Jan-2002, SOCS box proteins, unpublished). Then I found SSB-1 is more abundant in ovary tissue compared

to SSB-4 by the results of Northern blot, so I concentrated the expression pattern and functional work mainly on SSB-1 later on.

Materials and methods

Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from mouse ovary using Trizol reagent (GIBCO-BRL) according to the manufacturer's instructions. Single stranded cDNAs were prepared from 1 μg of RNA using MMLV reverse transcriptase (GIBCO-BRL) according to the manufacturer's instructions. The uncharacterized 5' and 3' regions of the mouse gene corresponding to the EST were obtained by rapid amplification of the cDNA ends (RACE) using 5'/3'RACE kit (Roche). This protocol required the production of four primers, a 3' RACE gene-specific primer and three 5' RACE gene-specific primers.

The gene-specific primers used to extend the 3' sequence was:

5'-GCACCCAGTAGCCCAGAGCA-3' (Gus5a).

The gene-specific primers used to extend the 5' sequence were:

5'-AAGGAATCTGGCAGAGCAAA-3' (Gus6),

5'-TAACCCACAGAGTGCAAAGG-3'(Gus7),

5'-TGCTCTAGGCTACTGGGTGC(Gus9).

The other primers used for series sequence of SSB-4 were:

5'-CCCTGCCTCAGTCTCTGAAA-3' (Gus4),

5'-TCCAGTTAGTTCCCCACTGC-3' (Gus31),

5'-AAAAGCACCTGGCCTTACCT-3' (Gus32).

Primers used for series sequence of similar to SSB-4:

5'-GTTTGACGTTTGGGACTGT-3' (Gus21),

5'-AACTCCTCAGTGGGCTGTTG-3'(Gus22),

5'-TGAATCCCAACCCTATGTCC-3' (Gus23)

5'-CAATTTCCGGGGTTCACTTTA-3'(Gus24).

PCR reactions were performed under 'hot-start' conditions with 5 min at 95°C and then 30 cycles of 45 sec at 95°C, 45 sec at 60°C and 2 min at 72°C. The 5' and 3' ends were subcloned using Zero Blunt TOPO PCR Cloning kit (Invitrogen). The PCR products and plasmids were sequenced at Université Laval.

Results and Discussion

5' RACE produced a product of approximately 800 bp, and 3' RACE produced several products ranging up to about 2 kb (Figure 2). When these products were sequenced, the 600 bp product of 3' RACE bore no similarity to the EST sequence, and was discarded as a non-specific product. The two ~2 kb products of the 3' RACE with the 800 bp product of the 5' RACE could, however, be assembled to generate longer cDNA sequences. One of these was 2553 bp and matches the cDNA later identified as *SSB-4* (Wang *et al.* 2005; Xing *et al.* 2006). The second was 2865 bp and was provisionally named *Similar to SSB-4* (*S-SSB-4*). *SSB-4* could encode a protein of 273 amino acids, and *S-SSB-4* a protein of 258 amino acids. The first 231 amino acids of these are shared. However, whereas SSB-4 includes the SPRY and SOCS box found in several other proteins, S-SSB-4 does not contain the SOCS box element. As well, the putative 3'untranslated regions located downstream of the translation termination signal of *SSB-4* and *S-SSB-4* are different (Figure 3).

Figures

Figure 1.

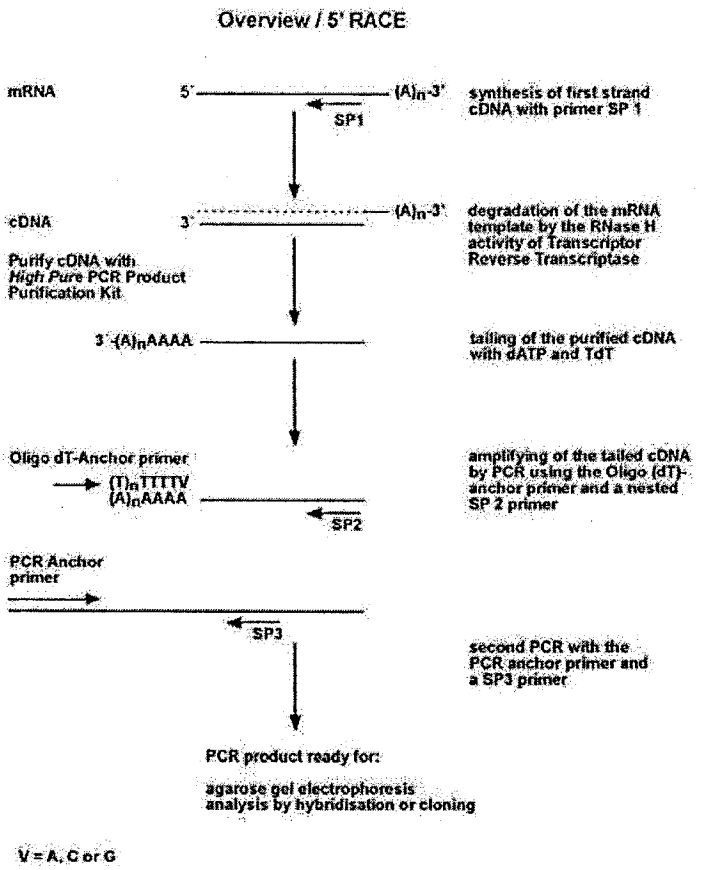
(A) The principle of 5' RACE. To generate the 5'-end, reverse transcription is carried out using a gene-specific primer (SP1). Then a poly(A) tail is added to the cDNA using terminal deoxynucleotidyltgransferase (TdT) and dATP. PCR amplification is carried out using the oligo-dT anchor primer attached to the added 5'-end and another nested gene-specific primer (SP2). A second round of PCR is carried out using the anchor primer and a third nested gene-specific primer (SP3). DNA sequencing is used to characterize the obtained 5'RACE products.

(B) The principle of 3' RACE. cDNA is synthesized beginning at the poly(A) tail of mRNA using the oligo-dT anchor primer. After converting mRNA into cDNA, PCR amplification is carried out with an anchor primer and a gene-specific primer (SP5). DNA sequencing is used to characterize the obtained 3' RACE products.

Figure 1 was taken from the Roche website.

Figure 1

A


Overview / 5' RACE
mRNA
5'
(A)n-3'
SP1
synthesis of first strand cDNA with primer SP 1
cDNA
3'
(A)n-3'
degradation of the mRNA template by the RNase H activity of Transcriptor Reverse Transcriptase
Purify cDNA with High Pure PCR Product Purification Kit
3'-(A)nAAAA
tailing of the purified cDNA with dATP and TdT
Oligo dT-Anchor primer
(T)nTTTTV
(A)nAAAA
SP2
amplifying of the tailed cDNA by PCR using the Oligo (dT)-anchor primer and a nested SP 2 primer
PCR Anchor primer
SP3
second PCR with the PCR anchor primer and a SP3 primer
PCR product ready for:
agarose gel electrophoresis analysis by hybridisation or cloning
V = A, C or G

B

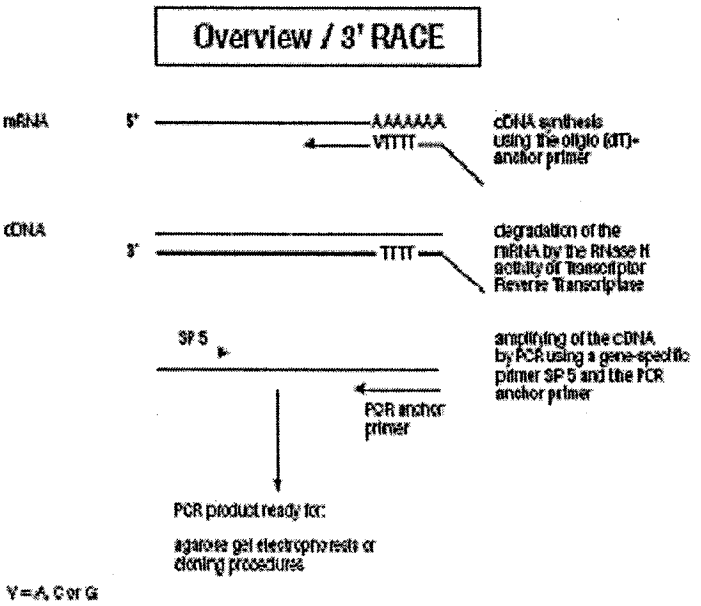

Overview / 3' RACE
mRNA
5'
AAAAAAA
VTTTT
cDNA synthesis using the oligo (dT)-anchor primer
cDNA
3'
TTTT
degradation of the mRNA by the RNase H activity of Transcriptor Reverse Transcriptase
SP 5
PCR anchor primer
amplifying of the cDNA by PCR using a gene-specific primer SP 5 and the PCR anchor primer
PCR product ready for:
agarose gel electrophoresis or cloning procedures
V = A, C or G

Figure 2.

Products generated by 5' (A) and 3' (B) RACE.

Figure 2

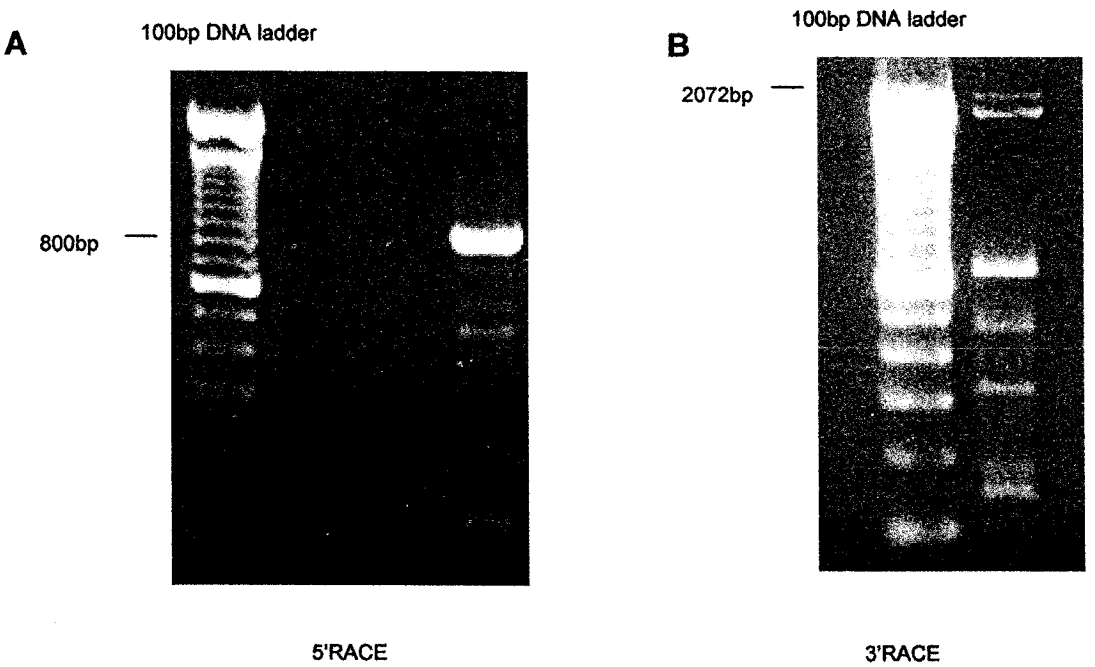

A
100bp DNA ladder
800bp
5'RACE
B
100bp DNA ladder
2072bp
3'RACE

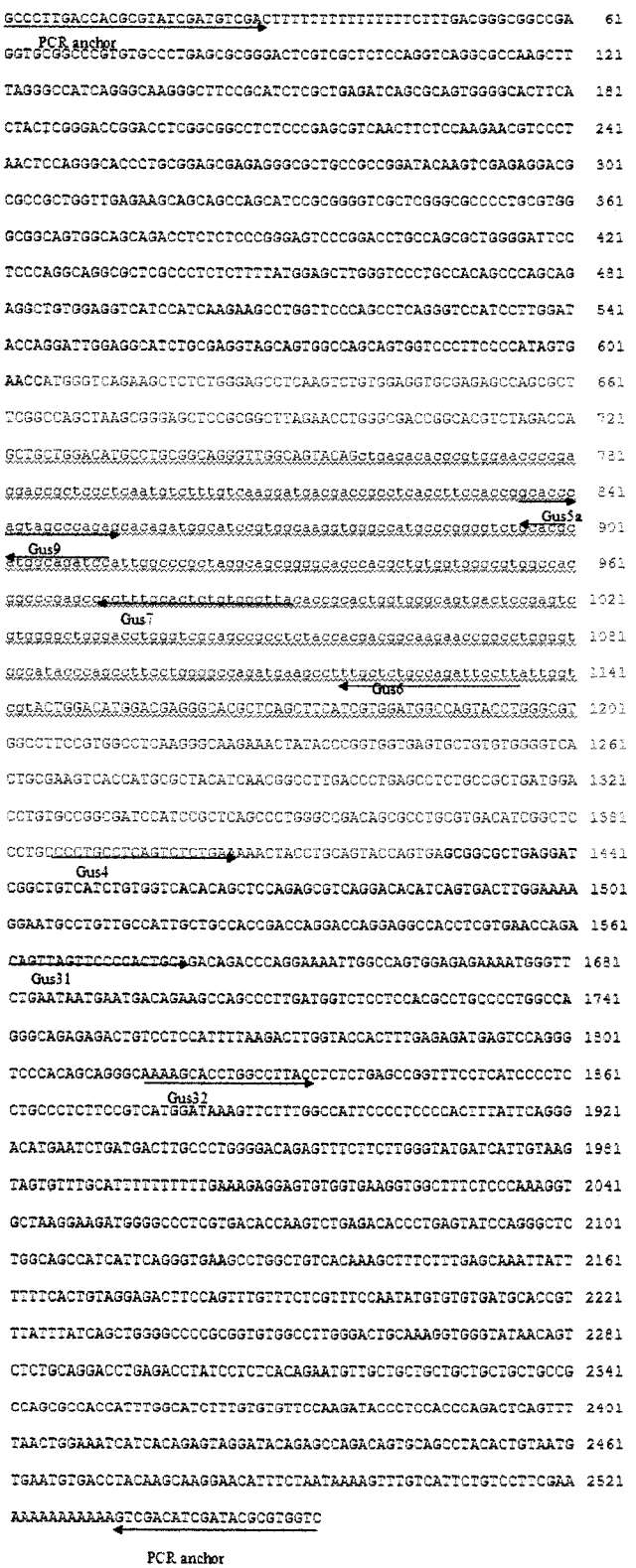
Figure 3.

Sequence of fragments obtained by RACE. (A) SSB-4. EST identified in database search is shown in red lower-case. Putative protein-coding region is shown in red. Putative 5' and 3' untranslated sequences are shown in black. Arrows indicate the primers used for RACE analysis. (B) S-SSB-4. Colours and arrows as for (A). (C) Comparison of SSB-4 and S-SSB-4. They share the 5'- untranslated region and most of the protein-coding region, but have different 3'-untranslated regions.

Figure 3

A

SSB-4



6. APPENDIX II

Making and purifying anti GST-fusion protein antibody for SSB-1 and SSB-4

Connecting text

Results of immunoblotting revealed that the anti-peptide antibodies for SSB-1 and SSB-4 not only recognized the predicted 31 kDa protein species in mouse ovarian tissue, but also recognized additional protein species. In the SSB-1 immunoblotting, there are another two bands at 40 and 36 kDa, and the SSB-4 antibody recognized an additional species at 60 kDa. In order to get specific staining pattern and provide a powerful tool for later functional research, I generated new antibodies raised against GST-fusion proteins. This appendix describes this work.

Introduction

SSB-1 and SSB-4 share 75% sequence similarity and both possess the SPRY and SOCS box domains that are present in other proteins. Therefore, to investigate the expression pattern of SSB-1 and SSB-4, it was decided initially to generate anti-peptide antibodies. The N-terminal 17 peptides of SSB-1 and SSB-4, in which SSB-1 and SSB-4 do not share very high sequence similarity to each other, were synthesized and injected into rabbit to make antibodies. Those anti-peptide antibodies recognized the corresponding SSB protein when it was over-expressed in cultured cells and neither recognized the other SSB protein. However, the SSB-1 antibody recognized additional protein species at 40 and 36 kDa, and the SSB-4 antibody recognized an additional species at 60 kDa (Xing *et al.* 2006). As it was not known whether these represented modified forms of the SSB proteins, it was decided to generate new antibodies.

As the first antibodies were raised against short peptide sequences, we chose to generate the second antibodies against a larger fragment of each SSB protein fused to a 'tag' to facilitate recovery. The best system in which to express such fusion proteins are eukaryotic cells, as these are able to modify proteins, such as by phosphorylation and glycosylation, to affect their antigenicity. Such modifications cannot be done by bacteria cells. Making and purifying polyclonal antibodies requires milligram amounts of purified protein, however, so this approach is not very practical. Therefore, it was decided to produce a GST-fusion protein in bacteria, because it is easy to get large amounts of purified protein from bacterial culture. As discussed above, the conserved regions of SSB-1 and SSB-4 lie mainly in the C-terminal portion (Xing et al., 2006). Therefore, the

DNA sequences encoding the N-terminal 60 amino acids of SSB-1 and the N-terminal 54 amino acids of SSB-4 were generated by PCR and inserted into plasmid PGEX-5X-1 and GST-fusion proteins were expressed in bacteria strain BL21. The proteins were purified with GST beads and purified proteins were used to immunize rabbits. After three months of standard immunization procedure, the serum was recovered and affinity-purified, and the affinity-purified antibodies were used for immunoblotting and immunostaining.

Materials and Methods

Animals

Experiments were carried out using CD-1 mice (Charles River, St-Constant, Quebec, Canada). All animal procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the McGill University Health Centre. Rabbit immunization and bleeding were performed by the McGill University Animal Resources Centre according to standard protocols.

Cloning of the DNA sequences encoding the N-terminal 60 amino acids of SSB-1 and N-terminal 54 amino acids of SSB-4

Total RNA was extracted from mouse ovary tissue using Trizol (Invitrogen) and reverse transcribed into cDNA using Moloney murine leukemia virus (MMLV) as previously described (Mohamed et al. 2004). cDNA obtained from 1 μg total RNA was subjected to PCR using a thermal cycler (Biometra UNO Thermoblock, version 3.30) using the following conditions: 95°C for 5 min and (94°C for 45 s, 50°C for 45 s, 72 °C for 1 min) x 35 cycles; the last cycle was followed by a 5-min extension at 72 °C. The primers used for amplifying SSB-1 N-terminal 60 amino acids are:

B-1-60a Forward 5'-CG**GGATCC**ATGGGTCAGAAGG-3' **BamHI**

B-1-60 Reverse 5'-CCG**GAATTC**TCAGTCGTTATTGTT-3' **EcoRI**

The primers used for amplifying SSB-4 N-terminal 54 amino acids are:

B-4-54a Forward 5'-CG**GGATCC**ATGGGTCAGAAGC-3' **BamHI**

B-4-54a Reverse 5'-CCG**GAATTC**TCAGTCCTCGGGGTT-3' **EcoRI**

The PCR fragments were purified using a commercial kit (Qiagen). The purified fragments and the PGEX-5X-1 plasmid were digested using the restriction enzymes BamHI and EcoRI (Invitrogen) according to manufacturer's instruction. Then the PCR fragments were ligated to the plasmid using T4 Ligase (Invitrogen) according to manufacture's instructions. The sequences of the newly generated plasmids were confirmed by DNA sequencing.

Generation of GST-fusion protein antibodies against SSB-1 and SSB-4

Expression and purification of GST-SSB protein

BL21 competent cells were transformed PGEX-5X-1 containing GST-SSB-1 or GST-SSB-4 according to standard protocols.

Small scale expression of fusion proteins

One single colony of GST-SSB-1 and GST-SSB-4 was picked and transferred to 3 ml of LB medium (with 100μg ampicillin) and the medium was shaken at 225 rpm for 5~6 hours until the OD600 = 0.5~0.6, taking 1 ml out as negative control. Then IPTG (0.5 mM) was added to the culture medium to induce protein expression. One ml culture medium was removed after 3 hours and centrifuged at 10,000 rpm for 5 min. The bacteria pellet was dissolved in 50 μl H2O or PBS, then combined with 10 μl of 6X SDS gel loading buffer and boiled at 95°C for 5 min. Proteins were separated by standard SDS-polyacrylamide gel electrophoresis (PAGE) and stained using 0.5% Coomassie blue R250 to verify the expression and size of the fusion protein.

Verification of protein solubility

Single colonies of transformants were picked and cultured in 10 ml LB medium overnight. The next day, 90 ml of fresh culture medium was added and the culture was continued for 2 to 3 hours until the OD600 = 0.5~0.6. The culture was centrifuged at 12,000 rpm for 30 min and the supernatant was discarded. The cell pellet was lysed with buffer (1X PBS, 1.5 mg/ml lysozyme, 1 mM PMSF, 1 tablet Roche proteinase inhibitor in every 50ml) and was subjected to three freeze/thaw cycles in liquid N_2 to rupture the cells. The cell lysate was then sonicated until it became water-like. The sonicated cell lysate was then centrifuged at 10,000 rpm for 30 min, and the supernatant and pellet were collected separately, dissolved in SDS gel loading buffer, and analyzed by SDS-PAGE. If the protein band is revealed to be in the supernatant, the GST-fusion protein would subsequently be purified using beads; if in the pellet, inclusive bodies would be isolated.

Large-scale expression of fusion proteins

Single colonies of transformants were picked and transferred into 250 ml LB medium containing 100 μg/ml ampicillin. The culture was grown overnight at 37°C with vigorous shaking. 2 L of LB medium containing 100μg/ml ampicillin was then added to this 250 ml and the culture was continued at 37°C with vigorous shaking until the OD600 = 1.0. IPTG was added to the culture medium to a final concentration of 0.1 mM to induce the protein expression. Then the culture was grown at 37°C with vigorous shaking for an additional 2 hours. The cells were harvested by centrifugation at 10000g for 30 min at 4°C. The supernatant was discarded and the pellets was stored at –80°C

Harvest and purification of fusion proteins

The cell pellets were resuspended in 30ml lysis buffer and incubated on ice for 30min. The cell lysate was frozen in liquid N_2 for 10min, then thawed rapidly in a 65°C water bath. This freeze/thaw cycle was performed three times until the solution became viscous due to the released bacterial DNA. The cell lysate was mixed frequently by inverting to avoid raising the temperature of the solution above 4°C during thawing. The viscous solution was then sonicated using a Sonicator 3000 (Misonix, Farmingdale, NY) on ice in short bursts of 20 seconds at mid to high power (5-6) 3 cycle and low power (2-3) 2 cycle until the viscosity of the solution was reduced to water-like. The solution was centrifuged at 8000rpm for 30min at 4°C and the supernatant was transferred to 50 ml tubes and stored at -80°C.

For protein purification, 1 ml of a 50% slurry of glutathione-agarose beads (Amersham, Montreal, QC, Canada) was added to 50 ml of lysate and the mixture was incubated with rocking at room temperature for 30 min, then at 4°C overnight. The mixture was centrifuged at 1000 rpm for 5min and the supernatant was removed. The supernatant was stored at –80°C. It can be reused when the large amounts protein are required. The beads were combined in one tube and washed six times with 1X PBS containing 1% Triton X-100, then three times with 1X PBS. The beads were put into poly-prep chromatography columns (Bio-Rad, Montreal, QC, Canada), and were washed twice with 1X PBS. The GST-SSB fusion proteins were then eluted from the beads at room temperature using 10 mM glutathione in 50 mM Tris-HCl, pH 8.0. Ten 1-ml fractions were collected in Eppendorf tubes. Five-µl samples from each tube were removed for protein quantitation.

The purified protein was dissolved at 1 mg/ml and used to immunize rabbits. Purified protein was stored at –80°C.

Antibody purification

The rabbit antisera were purified using Affigel-10 beads (Bio-Rad). Briefly, one ml of the 50% bead slurry was removed and washed three times with three ml of cold H_2O. The beads were transferred to a clean tube to which was added 5 mg of the GST-protein fragment used as immunogen. These were incubated overnight at 4°C with agitation to permit binding. The antigen-bound beads were loaded on poly-prep chromatography columns (Bio-Rad), which were then washed with 5 ml of H_2O, then with 10ml of antigen elution buffer AEB-1 (0.1 M glycine, pH 2.3), 10 ml of PBS, 10 ml of AEB-2 (50 mM triethanolamine, pH 11.5), and finally 10 ml of PBS. PBS-loaded columns were stored at 4°C until use.

Two ml of antiserum was diluted with 7 ml PBS and sodium azide was added to 0.02%. This was loaded onto the column, which was incubated at 4°C overnight with rotation. The column was washed with 10 ml PBS, then 10 ml PBS with 0.5 M NaCl, then 10 ml PBS again. Elution was performed with 10 ml AEB-1 into ten 1-ml tubes. Tris-HCl (pH 9.5) was added to each tube to neutralize the pH of the eluate. The column was washed with 10 ml PBS and stored at 4°C. The protein concentration in each collection tube was measured and the highest concentration was used for immunoblotting and immunostaining.

Immunoblotting

Ten ng of purified GST-SSB-1 and GST-SSB-4 were electrophoresed, transferred to PVDF membranes and immunoblotted as previously described. Primary antibodies were used at a dilution of 1:5000, secondary antibodies conjugated to horseradish peroxidase (HRP, Promega) at a concentration of 1:5000. HRP activity was revealed using ECL Plus (Amersham) following the manufacturer's directions.

Immunohistochemistry

Ovaries and testes were excised and fixed overnight at 4°C in freshly prepared 4% *para*-formaldehyde, washed in PBS, dehydrated and embedded in paraffin. Five-micrometer sections were exposed to 3% H_2O_2 in absolute methanol for 10 min, then washed three times in H_2O. The slides were placed in an antigen-retrieval solution (9 ml of 0.1 M citric acid and 41 ml of 0.1 M sodium citrate in 450 ml H_2O) and boiled for 10 min. After cooling, the slides were washed three times in PBS containing 0.1% Tween-20 (PBST). They were treated with a blocking solution (PBST, 3% BSA) for 1 h, then incubated overnight at 4°C with affinity-purified anti-GST fusion SSB-1 or SSB-4 diluted 1:500 in blocking solution. After three 30 min washes in PBST, the sections were incubated with fluorochrome-conjugated secondary antibodies (Jackson Immunoresearch, Molecular Probes) for 1 hour, slides were then mount with Glycerol. DNA was counterstained using DAPI (1 μg/ml for fluorescent microscope) or propidium iodide (10 μg/ml for confocal microscope). Alternatively, antibody binding was revealed by incubating the sections in biotinylated secondary antibodies (ABC kit, Vector Laboratories, Burlington, ON, Canada) for 1 hour, followed by washing and incubation with the ABC complex

according to the manufacturer's instructions. The sections were washed and stained with AEC color substrate (Vector Laboratories, Burlingame, Canada), then washed and counterstained with hematoxylin for 30 sec. Slides were mounted in glycerol and observed under brightfield microscope.

Results and Discussion

GST-fusion proteins containing the SSB-1 N-terminal 60 amino acids and SSB-4 N-terminal 54 amino acids can be expressed in bacteria

DNA fragments corresponding to N-terminal 60 amino acids of SSB-1 and the N-terminal 54 amino acids of SSB-4 were inserted into the GST-fusion expression plasmid PGEX-5X-1 (Figure 1) and the insertions were confirmed by DNA sequencing. The plasmids were used to transform the *E. coli* strain BL21 and expression was induced using IPTG. The predicted size of each fusion protein was approximately 33 kDa (GST tag: 27 kDa; 60 amino acids: 6 kDa). The GST-fusion proteins were purified from the bacterial lysate using beads. A single prominent band of the expected size was observed in each case, confirming expression of the proteins (Figure 2).

Anti-peptide antibody of SSB-1 and SSB-4 specifically recognize GST-SSB-1 and GST-SSB-4.

To further confirm the identity of the bacterially expressed protein, it was reacted with the anti-peptide antibodies. Both anti-peptide SSB-1 and anti-peptide SSB-4 recognized the corresponding GST-fusion protein and no cross-reaction was observed (Figure 3).

Antibodies raised against the GST-SSB-1 and GST-SSB-4 produce similar immunohistochemical staining patterns as the anti-peptide antibodies

The purified GST-fusion proteins were used to immunize rabbits and the antiserum was affinity-purified using corresponding GST-fusion proteins. Unexpectedly, the antibodies did not recognize SSB-1 or SSB-4 when it was expressed in cultured cells. This contrasts

with the anti-peptide antibodies, which reacted strongly with the proteins expressed in cultured cells. Therefore, the antibodies against the GST-fusion protein were not suitable for immunoblotting.

However, when these antibodies were used for immunohistochemistry on mouse ovarian tissue, these antibodies produced similar staining patterns as the anti-peptide antibodies (Figure 4). Both SSB-1 and SSB-4 were detectable in granulosa cells, including both mural and cumulus granulosa of antral follicles. SSB-1 and SSB-4 were also detected in the corpora luta, but not in the oocytes, thecal cells or interstitial tissue. On testis sections, they produced similar but not identical patterns as anti-peptide antibodies (Figure 5). In sexually mature animals, anti-GST-SSB-1 stained the spermatocytes, and possibly spermatogonia whereas spermatids and luminal sperm were only weakly stained. Anti-GST-SSB-1 also stained the cytoplasmic processes of the Sertoli cells that extend towards the lumen. This contrasts with the absence of Sertoli cell staining by the anti-SSB-1 peptide antibody. Similarly, anti-GST-SSB-4 stained the spermatocytes, whereas spermatids did not stain. Luminal sperm were strongly stained; however, this staining was also produced by the pre-immune serum and was therefore considered to be non-specific.

In neonatal animals, anti-GST-SSB-1 stained the cytoplasm of germ cells. Strong staining was observed in spermatogonia (large round cells in the middle of the seminiferous tubules), whereas weaker staining was present in the cytoplasm of the Sertoli cells (cells lying along the circumference of the seminiferous tubule) (Figure 6). Anti-GST-SSB-4

produced a staining pattern on neonatal testis similar to that of anti-GST-SSB-1 (data not shown).

Acknowledgements

I did this part of work in Dr. Paul Lasko lab. Thanks for the help and discussion from Dr. Han Hong.

Figures

Figure 1:

(A) Construction of plasmid PGEX-B-1-60aa encoding GST linked to the 60 N-terminal amino acids of SSB-1. DNA encoding the SSB-1 fragment was obtained by PCR using primers that included *Bam*HI and *Eco*RI restriction enzyme sites at ends of the PCR product. These were used to insert the fragment into PGEX-5X-1 plasmid. (B) Construction of GST-fusion SSB-4 plasmid PGEX-B4-54aa encoding GST linked to the 54 N-terminal amino acids of SSB-4. The strategy was the same as described for (A).

Figure 2:

Expression and purification of the GST-fusion proteins. (A) GST-SSB-1. *E. coli* strain BL21 were transformed using PGEX-5X-B-1-60aa and protein expression was induced by IPTG. Protein was purified using GST beads. The purified protein migrates near the predicted 33 kDa size. Lane1: Protein marker. Lane2: Crude BL21 lysate without IPTG induction. Lanes 3,4: IPTG induced SSB-1-60aa expression: 5μg total protein for Lane 3 and 10μg for Lane 4 . Lane 5: Supernatant of bacterial lysate. Fusion protein is present. Lane 6: Pellet of bacteria lysate. Fusion protein is not detectable. Lanes 7: Purified GST-SSB-1 20 ng (B) GST-SSB-4. The methodology and lane identifications are as described for (A).

Figure 3

Detection of GST-SSB-1 and GST-SSB-4 using corresponding anti-peptide antibodies. Ten ng of GST-purified fusion protein was electrophoresed and analyzed by immunoblotting using either the anti-B-1 peptide antibody 3314 (A) or the anti-B4 peptide antibody 3311 (B). Both GST-SSB-1 and GST-SSB-4 are recognized by the corresponding anti-peptide antibody but not by the non-corresponding anti-peptide antibody.

Figure 4

Immunostaining of mouse ovarian sections with anti-GST fusion protein antibodies. Ovaries were fixed in 4% *para*-formaldehyde, embedded in paraffin and sectioned at 5-μm thickness. Sections were subjected to an antigen-retrieval technique. (A) SSB-1 is detected in granulosa cells, including both mural and cumulus of antral follicles. SSB-1 is also detected in the corpus luteum, but not in the oocytes, thecal cells or interstitial tissue. (B) SSB-4 is detected in the granulosa cells in follicles at different stages of growth. Scale bar: 100 μm.

Figure 4

A

B

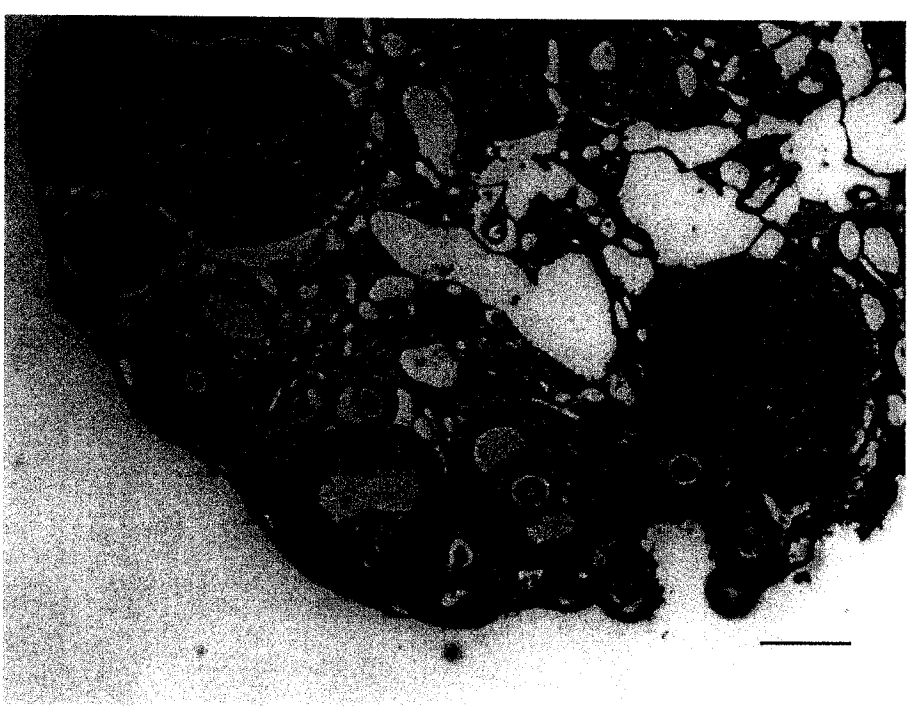
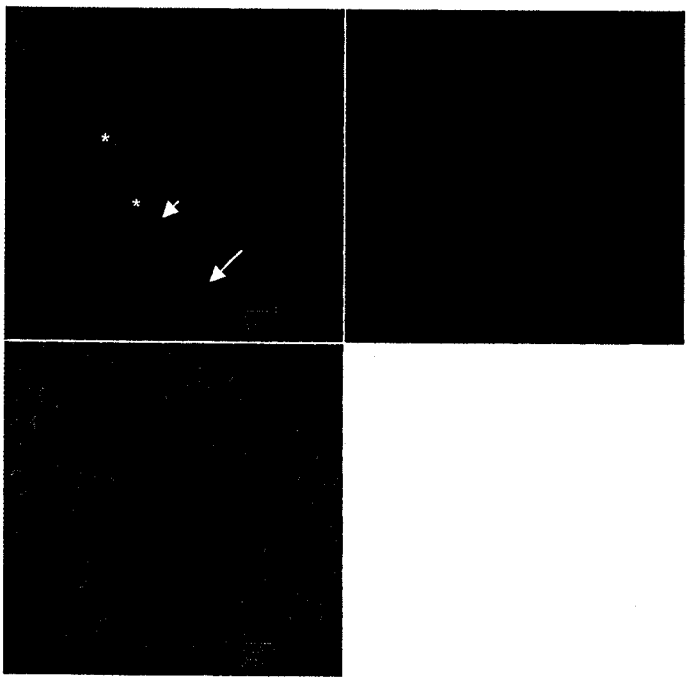


Figure 5

Immunostaining of testis sections of mature mice with anti-GST fusion protein antibodies. Testes were fixed in 4% *para*-formaldehyde, embedded in paraffin and section at 5-μm thickness. Sections were subjected to an antigen-retrieval technique. (A) Anti-GST-SSB-1 stained the spermatocytes (arrow) and possibly spermatogonia whereas spermatids (arrowhead) and sperm were only weakly stained. Sertoli cell cytoplasmic processes (asterisk) were also stained. (B) Anti-GST-SSB-4 stained the spermatocytes (arrow) whereas spermatids (arrowhead) did not stain. Luminal sperm (asterisk) also stained strongly, but this staining was also observed using pre-immune serum and was considered to be non-specific. Scale bar: 20μm. Upper left photos of A and B are SSB-1 and SSB-4 staining. Upper right photos are DNA stained by propidium iodide. Lower are merged photos of A and B.

Figure 5

A



B

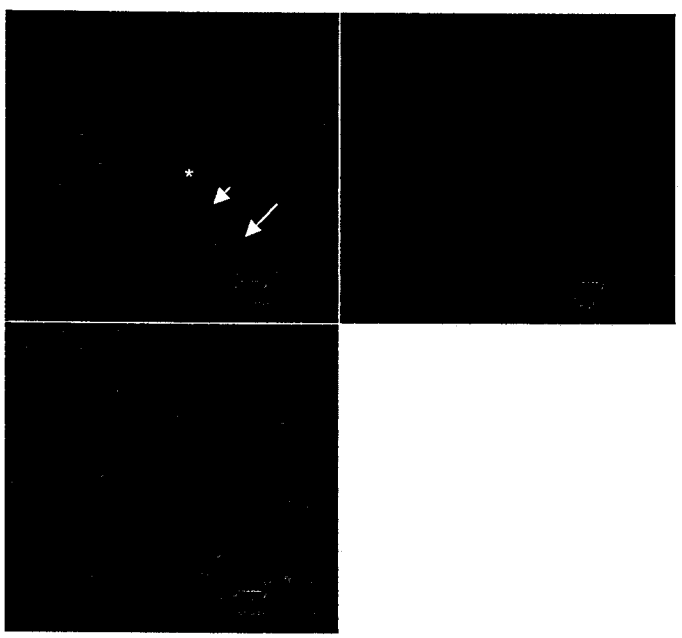
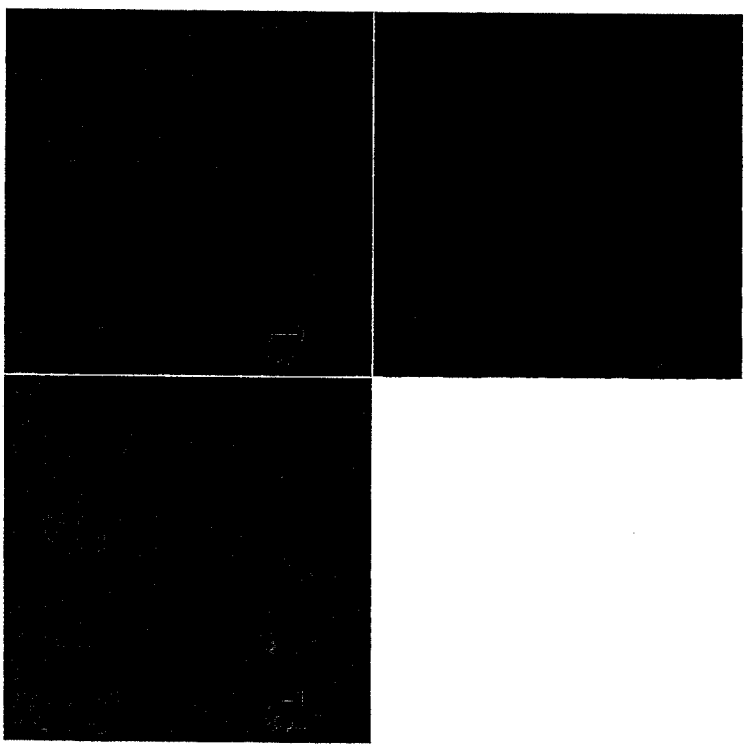


Figure 6

Immunostaining of neonatal testis sections with anti-GST-fusion SSB-1 antibody. Testes were fixed in 4% *para*-formaldehyde, embedded in paraffin and sectioned at 5-μm thickness. Sections were subjected to an antigen retrieval technique. Anti-GST-SSB-1 stained the cytoplasm of germ cells. Strong staining was observed in the cytoplasm of spermatogonia (large round cells in the middle of the seminiferous tubules). Weak cytoplasmic staining was present in Sertoli cells (lying along the circumference of the seminiferous tubule). Scale bar: 20 μm. Upper left photo is staining of SSB-1 antibody. Upper right photo is DNA stained by propidium iodide. Lower is merged photo of A and B.

Figure 6



7. GENERAL DISCUSSION

In this thesis murine homologues of the *Drosophila* Gustavus gene was identified; namely, SSB-1 and SSB-4. Next, the expression patterns of SSB-1 and SSB-4 in mouse ovarian and testis tissue was described. Finally, the function of SSB-1 was investigated using the RNAi technique.

1. Screen ovarian library to see if there are more *gustavus* relative genes in mouse genome.

This project was began by searching a database. Searching the mouse GenBank with the *Drosophila* GUS protein and a short EST was obtained. Then, 3'/5' RACE was used to obtain the sequence of SSB-4 and a gene that I termed, Similar to SSB-4. The sequence of SSB-1 was posted on the NCBI website (http://www.ncbi.nlm.nih.gov/entrez/) one year after this project was begin. Similar to *Drosophila* GUS, both SSB-1 and SSB-4 belong to a family of proteins that contain both a SPRY domain and a SOCS box. Only four members were found in this family have been identified. The other two, SSB-2 and SSB-3, are not similar to GUS outside these domains. It would be of interest to undertake further screening, especially of ovarian cDNA libraries, to see whether there are more *gus*-related genes in the mouse genome.

2. Multiple bands of SSB-1 antibody

To study the pattern of expression of SSB-1 and SSB-4, four antibodies were made and tested. Sequence alignment of SSB-1 and SSB-4 revealed very high sequence similarity

even outside the SPRY and SOCS domains. Anti-peptide antibodies were firsted to be make against the seventeen N-terminal amino acids where SSB-1 and SSB-4 sequence are not similar. When the affinity-purified antibodies were tested on ovarian and testis tissues, both SSB-1 and SSB-4 antibodies were found to be recognize by multiple bands. Anti-SSB-1 antibody recognized three protein species at 40, 36 and 31 kDa. Among these, the 31kDa band is the same as the theoretical predicted size and also migrates in the same position as the SSB-1 in transfected cell lines. Moreover, only the 31kDa species could be immunoprecipitated by the SSB-1 antibody.

To obtain antibodies showing more specificity for the 31 kDa SSB-1, GST-fusion protein antibodies against sixty N-terminal amino acids of both SSB-1 and SSB-4 were made. Unexpectedly, the affinity-purified GST-fusion protein antibodies did not recognize the corresponding protein when they were expressed in immortalized cell lines and could not be used to immunoprecipitate the proteins. However, the immunostaining patterns of those antibodies on ovarian tissue were the same as the anti-peptide antibodies. This suggested that the antibodies recognized a structurally complex epitope. Future approaches could focus on making polyclonal antibodies in another species such as chicken or pooled monoclonal. These strategies were not feasible, however, in the context of this research project.

Then the specificity of the immunostaining pattern might be doubted. In the first manuscript, all experiments from Northern blot, in situ hybridization, RT-PCR and western blot reveal that the main expression site for SSB-1 is different stages of granulosa

antibodies and anti-GST fusion protein antibodies give the same staining pattern. Therefore, the conclusion that SSB-1 and SSB-4 are localized in different stages of granulosa cells seems justified. In the second manuscript, results of western blot showed that the expression patterns of SSB-1 in testis sertoli cells and germ cells are similar to that was found in ovary and granulosa cell line. In order to confirm the immunostaining patterns on testis, results of *in situ* hybridization on testis section would be helpful. An accurate staining pattern could be obtained by making GFP-SSB-1 transgenic mice. However, this is a very time-consuming procedure and not feasible in the context of this research project.

3. Do MVH (murine Vasa homologue) and SSB-1 interact with each other in mouse tissue ?

In *Drosophila,* VASA and GUS interact both in *vivo* and in *vitro* and both play important functions in translational regulation. This interaction is the theoretical foundation of this thesis. The phenotype of MVH knockout mice presented a surprise (Tanaka *et al.* 2000). Unlike Drosophila VASA, MVH did not function during mouse oogenesis. VASA protein is present only in small oocytes, disappearing as the oocytes grow. SSB-1 and SSB-4 did not localize to mouse oocytes; rather, their main expression site is granulosa cells instead. Since MVH is not expressed in granulosa cells, these two proteins must not interact with each other in mouse ovarian tissue.

Homozygous knockout MVH male mice are infertile. In the mature testis, MVH protein is expressed in the cytoplasm of spermatogenic cells and chromatoid bodies in

spermatids. SSB-1 also is found to be express in the cytoplasm of spermatocytes and chromatoid bodies. The same localization of SSB-1 and MVH led me to test whether they interact with each other in mouse testis. Co-immunoprecipitation was performed with testis tissue and shows that MVH and SSB-1 antibodies did not co-immunoprecipitate the other protein (data not shown). They also did not co-immunoprecipitate in a cell line expressing their full length coding regions. However, that results might be not real because, as we presented previously, the antibodies we used is not very strong for SSB-1 in cell line. The 31 kDa band is relatively weak in mouse ovarian and testis tissue. These antibodies might not strong enough to co-immunoprecipitate the interaction protein. Alternatively, in a multi-protein complex, the antigenic site might be inaccessible to the antibodies. GST pull-down assay could be used to further test the interaction. Making the full-length GST fusion protein of VASA and SSB-1, once the interaction was found to be positive, different fragments of GST fusion protein could be made to identify the interaction site. Another approach is to test whether MVH and SSB-1 interacted with each other in vitro using the yeast two-hybrid system.

4. Does SSB-1 localize in the mitochondria?

A major site of SSB-1 expression in mouse is the granulosa cells. Subcellular localization of SSB-1 was tested. The protein localization is usually related to its function. For example, SSB-1 localizes to the chromatoid body in mouse speramtids. *Drosophila* GUS and VASA also localize to perinuclear ribonucleoprotein particles in nurse cells and pole plasm in oocytes. VASA homologue in *Xenopus* localizes to the mitochondria cloud (Komiya *et al.* 1994; Tanaka *et al.* 2000). I then found that P450scc, the first and rate-

limiting steroidogenic enzyme that is localized in mitochondria, decreases when SSB-1 is depleted. Therefore it is very interesting to test if SSB-1 localized in mitochondria. Different organelles were separated by step centrifugation. I found SSB-1 to be present in all different fractions. SSB-1 might be secreted protein, so all different portions of organelles have contamination. Thus, this question merits further attention.

5. Why RNAi did not work on cultured granulosa-oocyte complexes

For functional studies of SSB-1, SiRNA-based techniques were used. The physiological state of granulosa cells depends on the oocyte, in that following ovulation or other treatment that remove the oocyte from the environment, the granulosa cells undergo luteinization. RNAi on cultured oocyte-granulosa cell complexes was firsted to be tested, because the physiological state of granulosa cells in this cultured complex is close to the in vivo situation.

SiRNAs were labelled with Cy3 and transfected labelled siRNA using different transfection reagents. Then the transfection efficiency tested with flow cytometry. Depsite very high transfection efficiency, no transfection reagent could decrease the SSB-1 RNA level by more than 20%. Different culture mediums (MEM and Waymouth, with FBS or BSA), different types of collagen dishs (12 well, 6 well)were tested – all of them gave similar results. Different concentrations of siRNA were also tested, from 10 nM to 200 nM. All of them gave similar results until, at 200 nM, all complexes degenerated 24 hr after transfection.

Electroporation was then tested on the cultured complexes. Different electroporation buffers were tested at different voltages and frequency. None could bring the fluorescent siRNA into the cultured complexes until the conditions are too hard to cause complexes degeneration. Therefore, we concluded that the siRNA can be brought into the cytoplasm of granulosa cells in the cultured GOCs. But the lipo-siRNA complex might not be released so the siRNA cannot perform its function in the cultured complexes. We then tested siRNA on immortalized cell lines and got ideal results.

6. RNAi on mouse granulosa cell line.

RNAi effectively reduces SSB-1 levels in the mouse granulosa cell line, NT-1. For phenotype analysis, I screened the expression levels of several genes important for granulosa cell function and expressed abundantly in NT-1 cells (Rilianawati *et al.* 1999), including *FSHR*, *LHR*, *P450scc*, P450 aromatase, and *KL*. *FSHR*, *LHR* and P450 aromatase could not be detected in this cell line, that might caused by late passage number. *P450scc* and *KL* level decreased when SSB-1 was depleted. Using a P450scc antibody, I found P450scc protein level also decreased in the treated cells.

Real-time RT-PCR was used to test gene expression level. It is a very efficient and accurate method to measure RNA quantities in the tested samples, but the number of samples that can be tested is limited by this method. DNA chip analysis is then considered. However the DNA chip is not as sensitive as real-time RT-PCR unless a granulosa cell-specific DNA chip was obtained. We can then run this cell type-specific chip and compare all the gene expression levels in the normal NT-1 cells to SSB-1 knock-

down NT-1 cells. With this method, more potential targets will be found. Once the downstream target genes of SSB-1 are found, relevant antibodies can be used to confirm the RNAi results.

In mouse testis, SSB-1 is expressed in the cytoplasm of Sertoli cells and in germ cells, notably in the chromatoid body. It is promising to investigate the function of SSB-1 in spermatogenesis. One approach would be to first try RNAi on immortalized spermatocyte cell lines and Sertoli cell lines. Once RNAi is found to be working, the phenotype can be analyzed by real-time RT-PCR. Antibodies can be used to confirm the RNA data.

On the other hand, recent publications show new RNAi methods on spermatogenesis (Komiya *et al.* 1994; Tanaka *et al.* 2000; Shoji *et al.* 2005). DNA vectors that encoded small hairpin RNAs were electroporated *in vivo* into the testis. Sequence-specific reduction in the reporter products was confirmed, and the same phenotypes were observed in null-mutant mice. This novel rapid method could be used to investigate the function of SSB-1 in mouse spermatogenesis.

7. Future direction of this project

In order to know clearly the function of SSB-1, the most direct and elegant approach is to make SSB-1 null mutant mice. Since this technique requires a long period of time, this goal could not be achieved during the period of my doctoral research. But, with the understanding of the expression pattern of SSB-1 and its possible function, making knockout mice in the long term is promising.

SSB-1 is expressed in many different mouse tissues and also in mouse embryo, so conventional knockout might cause embryo lethal. SSB-1 could be depleted using a Cre-loxP conditional knockout. After generating mice carrying a floxed allele of SSB-1, this line could be crossed with another mouse line expressing the Cre recombinase under the control of a granulosa cell promoter which is only active in different stages of granulosa cells. So far, the *Amhr2* promoter has been used to make granulosa cell-specific Cre mice (Jorgez *et al.* 2004); the *Amh* promoter was used to make Sertoli cell-specific Cre mice (De *et al.* 2004a); the *TNAP* promoter was used to make spermatogonia-specific Cre mice (Kaneda *et al.* 2004).

SSB-4 has very high percentage of sequence similarity to SSB-1. I concentrated my research on SSB-1 because SSB-1 is much more abundant compared to SSB-4. According to the results of real-time PCR of cultured granulosa-oocyte complexes, SSB-1 expression level is 8-fold higher than SSB-4. But this does not exclude the possibility that SSB-4 might also have an important physiological significance. RNAi can be used to test the function of SSB-4. If the phenotype is similar to that found when SSB-1 is depleted, this might indicate functional redundancy of SSB-1 and SSB-4. In this case, making knock-out mice for SSB-4 and double knock-out mice for B-1 and B-4 might be considered in future research.

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