EXPRESSION AND FUNCTION OF BASIGIN DURING EARLY PREGNANCY AND SPERMATOGENESIS

BY

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DISSE TATION

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Abstract

Basigin (BSG) is a multifunctional glycoprotein that plays an important role in both female and male reproduction since female knockout (KO) mice are infertile and male KO mice are sterile. The aim of the present study was to determine 1) whether BSG is required for proliferation of the uterine luminal epithelium during early pregnancy in preparation for implantation; 2) whether BSG is required for HESCs decidualization; and 3) whether BSG is essential for the interactions between gametes and Sertoli cells during spermatogenesis.

BSG protein was expressed in the uterine epithelium at estrus in βERKO mice but not in αERKO mice. However, a higher level of Bsg mRNA was observed in the uteri of αERKO mice as compared with wild type (WT) and βERKO mice. In the mouse, estrogen alone induces the proliferation of both luminal and glandular epithelial cells during early pregnancy. On day 1 of pregnancy, the expression levels of ERα and a well-known estrogen responsive gene, MUC1, appeared to be normal in the uteri of Bsg KO females. This suggested that the circulating estradiol levels in Bsg KO mice are normal. I examined proliferation in uterine epithelial cells and found that, in WT mice, uterine epithelial cells were highly proliferative as measured by expression of Ki67 in both the luminal and glandular epithelial cells. In contrast, Ki67 expression was significantly decreased in the epithelial cells of Bsg KO uteri. Immunochemistry using the BSG antibody revealed that the expression of BSG in C/EBPβ null uteri was heterogeneous and spotty in the luminal epithelium as compared with a more homogeneous expression pattern of BSG in the uterine epithelium of WT uteri. These results indicate that C/EBPβ may be one of the factors regulating the expression of BSG
Decidualization of human endometrial stromal cells (HESCs) into decidual cells is carefully controlled, both spatially and temporally, by a balance of stimulatory and inhibitory signals from the uterine endometrium and is a prerequisite for successful implantation. Decidualization is associated with induction of a number of genes including wingless-type MMTV integration site family member (WNT4) and forkhead transcription factor forkhead box O1A (FOXO1). In the present study, HESCs were transfected with small interfering RNAs targeting BSG gene expression. Expression of the decidualization markers Insulin-like growth factor-binding protein 1 (IGFBP1) and Prolactin (PRL) was significantly inhibited in cells with down regulated BSG expression. Silencing of BSG in HESCs also impaired expression of several MMPs. Microarray analysis revealed that both WNT4 and FOXO1 and its downstream targets are under the regulation of BSG during decidualization in HESCs.

The Bsg KO testis lacks elongated spermatids and mature spermatozoa, a phenotype similar to that of alpha-mannosidase IIx (MX) KO mice. MX regulates formation of N-acetylglucosamine (GlcNAc) terminated N-glycans that participate in germ cell-Sertoli cell adhesion. Results showed that Bsg KO spermatocytes displayed normal homologous chromosome synapsis and progression to the midpachytene stage. Both meiosis I (MI) and meiosis II (MII) spermatocytes were detected in the KO preparations. However, expression of the acrosome marker SP-10 was extremely low in germ cells of Bsg KO mice indicating that spermatogenesis in Bsg KO mice was arrested at the round spermatid stages. I observed a large increase in the number of germ cells undergoing apoptosis in Bsg KO testes. Using lectin blotting, I determined that GlcNAc terminated
N-glycans are linked to BSG. GlcNAc terminated N-glycans were significantly reduced in Bsg KO testes. These observations indicate that BSG may act as a germ cell-Sertoli cell attachment molecule. Loss of BSG significantly reduced adhesion between GC-2 and SF7 cells. Moreover, WT testes showed strong expression of N-cadherin (CDH2) while expression was greatly reduced in the testes of Bsg KO mice. In addition, the integrity of the blood-testis barrier (BTB) was compromised and flow of fluid from the testis was reduced in Bsg KO testes.

My findings suggest that expression of BSG protein in the uterus requires estrogen acting through ESR1, but not ESR2. Moreover, estrogen may regulate the proliferation of both luminal and glandular epithelial cells in the uterus during early pregnancy through a pathway involving BSG. Human decidualization results have provided new insights into the molecular pathways that regulate decidualization of human uterine stromal cells. Understanding the role of BSG during decidualization may help to explain the defects in decidualization-associated reproductive disorders of women. Male results indicate that although Bsg KO spermatogonia can undergo normal progression to the spermatocyte stage, BSG-mediated germ cell-Sertoli cell adhesion via GlcNAc terminated N-glycans appears to be necessary for integrity of the BTB and spermatocyte progression to mature spermatozoa.
I dedicate this dissertation to Lord for giving me faith and guidance throughout my life.

I also dedicate this to my grandmother, my parents, my wife and my two lovely kids for whom without their love and support I would have never found the strength to finish my Ph.D.
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# Table of Contents

List of Tables .................................................................................................................. viii  
List of Figures .................................................................................................................. ix  
Chapter 1: Introduction and Rationale ........................................................................... 1  
Chapter 2: Literature review ........................................................................................... 3  
  • A. Basigin (BSG)....................................................................................................... 3  
  • B. Implantation ......................................................................................................... 12  
  • C. Spermatogenesis .................................................................................................. 19  
  • D. Biosynthesis of N-glycan Carbohydrates in Mammalian Cells ......................... 20  
Chapter 3: BASIGIN is Essential for Sensitizing the Uterine Luminal Epithelium in Mice During Early Pregnancy ................................................................. 33  
Chapter 4: Basigin is Necessary for Normal Decidualization of Human Uterine Stromal Cells ................................................................................................................. 60  
Chapter 5: Sterility in Basigin Null Mutant Male Mice May Be Due to Impaired Interactions Between Gametes and Sertoli Cells .............................................. 86  
Chapter 6: Summary of Results and Future Directions ................................................ 128  
Chapter 7: Materials and Methods ................................................................................. 133
List of Tables

4.1. Silencing of BSG expression in decidualizing HESCs leads to down regulation of MMP expression ................................................................. 80

4.2. Silencing of BSG expression in decidualizing HESCs leads to down regulation of WNT and FZD family members expression ....................... 81

4.3. Silencing of BSG expression in decidualizing HESCs leads to down regulation of expression of FOXO1 and its target genes ............................ 82

7.1. The procedure of tissue process ............................................................................................................................... 150

7.2. List of primary antibody ........................................................................................................................................ 151
List of Figures

2.1. The structure of basigin .......................................................... 22
2.2. Window of receptivity in the mouse ........................................ 23
2.3. Structure of the three major types of N-glycans ....................... 24
3.1. BSG expression in uteri of αERKO and βERKO mice ............... 49
3.2. BSG protein levels in uteri of WT and ERKO mice ................. 50
3.3. Bsg mRNA levels in uteri of WT and ERKO mice .................. 51
3.4. Ki 67 immunoreactivity in the uteri of WT and Bsg KO mice on day 1 of pregnancy ........................................................... 53
3.5. ERα, MUC1 immunoreactivity in the uteri of WT and Bsg KO mice on day 1 pregnancy ................................................................. 54
3.6. Ki67 immunoreactivity in the uteri of ovariectomized WT and Bsg KO mice treated with 200ng estradiol ........................................ 55
3.7. BSG immunoreactivity in the uteri of ovariectomized WT and C/EBPβ KO mice treated with 250ng estradiol ................................ 56
4.1. Immunohistochemical analysis of BSG protein localization during pregnancy ................................................................. 75
4.2. Silencing of BSG expression inhibits stromal cell proliferation .... 76
4.3. Silencing of BSG expression inhibits decidualization ............... 77
4.4. Silencing of BSG expression reduces MMP production ............ 78
4.5. BSG-WNT4-FOXO1 pathway in HESC decidualization ............ 79
5.1. Abnormalities in mouse testes due to loss of BSG expression .... 111
5.2. Surface-spread chromatin preparations documenting normal chromosome pairing, recombination and chromatin remodeling in WT and Bsg KO spermatocytes .................................................. 113
5.3. Localization and quantitation of SP-10 protein in testes of WT and
5.4. Abnormalities in testes of aged Bsg KO mice ................................................................. 116
5.5. BSG immunoreactivity in mouse testes ............................................................................. 117
5.6. Integrity of the BTB and localization of BTB related adhesion molecules CXADR, CX43, CLDN11 and CDH2 in the testes of WT and Bsg KO mice ......................................................................................... 118
5.7. Localizations of GlcNAc terminated N-glycans in the testes of WT and Bsg KO mice ........................................................................................................ 120
5.8. GlcNAc-terminated N-glycans are one of the carbohydrates linked to BSG ........................................................................................................ 121
5.9. Adhesion of GC-2 spermatocyte cells transfected with Bsg siRNA to SF7 Sertoli cells ........................................................................................................ 122
Chapter One
Introduction and Rationale

Basigin (BSG), a member of the immunoglobulin superfamily, is a highly glycosylated transmembrane protein with two immunoglobulin-like domains (Miyauchi et al., 1990; Seulberger et al., 1992). BSG is expressed abundantly both in the uterus and testis in mice (Chen et al.; Igakura et al., 1998). In the mouse uterus, BSG is highly expressed both in the luminal and glandular epithelium on day 1 and day 2 of pregnancy. Beginning at day 4 of pregnancy, BSG is detectable in the uterine stromal cells (Chen et al., 2009). In the mouse testis, BSG is expressed in Sertoli cells, Leydig cells and all germ cells (Chen et al., 2010). Female Bsg knock out (KO) mice are infertile and males are sterile; female mice due to implantation defects during early pregnancy and males due to the failure of spermatogenesis (Igakura et al., 1998). Thus, it is clear that BSG is required in early pregnancy and during spermatogenesis. The central hypothesis of this study is that BSG is required for both female and male reproductive processes. The specific hypotheses that were investigated in this dissertation were:

1) BSG is required to prepare the luminal epithelium for embryonic attachment during early pregnancy.
2) BSG is necessary for normal decidualization of human uterine stromal cells (HESCs).
3) BSG is essential for the interactions between gametes and Sertoli cells during spermatogenesis.

The overall objective of this dissertation was to understand the role of BSG in females in early pregnancy and in males during spermatogenesis. In Chapter 2, an overview of
BSG, implantation/decidualization and spermatogenesis is presented. Chapter 3 discusses my findings on how BSG plays a role in early pregnancy in mice. The role of BSG in decidualization of HESCs is discussed in Chapter 4. My findings on the role of BSG during spermatogenesis in mice are presented in Chapter 5. Chapter 6 contains a summary of my findings and directions for future studies. Lastly, the appendix includes detailed information on the materials and techniques used in all experimental studies.
A. Basigin (BSG)

A.1 History of BSG

In the 1980s Dr. Biswas’ group co-cultured rabbit fibroblasts with mouse tumor cells of epithelial origin and found that these showed increased collagenase production when compared to cultures of fibroblasts alone (Biswas, 1982). Later they achieved similar results when they co-cultured human fibroblasts with 3 different human tumor cell lines. The tumor cell lines were found to secrete a protein(s) that stimulates collagenase production by human fibroblasts (Biswas, 1984; Biswas and Nugent, 1987). This protein was purified from human lung carcinoma cells and was named TCSF (tumor cell collagenase-stimulatory factor). Immunofluorescence analysis indicated that TCSF was present on the tumor cell surface (membrane protein) and was important in inducing collagenolysis of tissue stroma during tumor invasion (Ellis et al., 1989). Subsequently it was found that TCSF could stimulate not only the production of interstitial collagenase (MMP-1) in human fibroblasts, but also of fibroblast-derived stromelysin-1 (MMP-3) and 72- kDa gelatinase/type IV collagenase (MMP-2) (Kataoka et al., 1993). TCSF was then renamed as Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) to indicate its role in extracellular matrix metalloproteinase induction in normal, as well as pathological, cellular interactions (Biswas et al., 1995). Several different labs independently identified this protein in several different species. Thus EMMPRIN is also known as CD147 and M6 in human, BSG and gp42 in mice, HT7 and neurothelin in chicken and OX-47, MC31 and CE9 in the rat (Nehme et al., 1995; Sameshima et al.,
2000; Schlosshauer and Herzog, 1990; Seulberger et al., 1992; Wakayama et al., 2000). Because my studies focus primarily on the function of EMMPRIN in the mouse, the name BSG will be used in this proposal.

**A.2 Structure of BSG**

Basigin (basic immunoglobulin superfamily) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily (Miyauchi et al., 1990). The molecular weight of BSG lies largely in a range between 43 kD and 66 kD due to variable glycosylation. Deglycosylation of BSG by endoglycosidase treatment leads to a reduction of the molecular weight to about 27 kD (Fadool and Linser, 1993; Schlosshauer, 1991). The extracellular domain of BSG contains four cysteines. These four cysteines organize the extracellular region of BSG into two immunoglobulin-like domains by forming two disulfide bonds (Fossum et al., 1991; Kasinrerk et al., 1992) (Fig. 2.1). BSG is highly conserved among species. Amino acid sequence analysis indicates that there are five potential N-linked glycosylation sites in the extracellular domain of chicken BSG. Three of five potential N-glycosylation sites are conserved in human, mouse and rat. Furthermore, the transmembrane region is also highly conserved across species. Human, mouse, rat and chicken BSG show almost 100% amino acid sequence identity in this region. The high degree of conservation in the transmembrane and cytoplasmic regions suggests an important functional role for these domains of the protein (Fossum et al., 1991; Kasinrerk et al., 1992; Seulberger et al., 1992).
To date four different human isoforms of BSG have been identified. BSG isoform 1 has three Ig-like domains and is a retina-specific isoform (Hanna et al., 2003; Ochrietor et al., 2003). The prototypical isoform, BSG isoform 2, is expressed in most tumor and fibroblast cells and is also the only isoform that is secreted (Belton et al., 2008; Liao et al., 2011). BSG isoforms 3 and 4 are less abundant and were first identified in human endometrial stromal cells and cervical carcinoma cell lines. Glycosylated BSG isoforms 3 and 4 are expressed and localized to the plasma membrane in various normal human tissues at the mRNA level. Although the functions of BSG isoforms 3 and 4 are still unclear, it has been shown that soluble BSG is rapidly internalized after binding to BSG isoform 2 in the cell membrane, resulting in a subsequent interaction between the ligand and BSG isoform 3 protein (Belton et al., 2008). Moreover, BSG isoform 3 has been shown to inhibit hepatocellular carcinoma cell proliferation, MMP induction, and cell invasion (Liao et al., 2011).

A.3 BSG and its Signaling Pathway

BSG acts through several different signaling pathways. Studies have shown that BSG acts as its own receptor at the cell surface, resulting in the activation of the ERK1/2 signaling pathway leading to increased expression of matrix metalloproteinase-1 (MMP-1), MMP-2, and MMP-3 in uterine fibroblasts (Belton et al., 2008). BSG also acts as a cell surface receptor for cyclophilin A (CyPA) and is an essential component in the CyPA-initiated signaling cascade that culminates in ERK activation (Yurchenko et al., 2002). CyPA is an ubiquitously expressed intracellular protein and plays a role in protein folding. It is also secreted by cells during inflammation (Sherry et al., 1992). In addition,
it has been shown that BSG can stimulate the production of MMP-1 through p38 MAP kinase in human lung fibroblasts. Blocking p38 with an inhibitor significantly decreases the production of MMP-1 (Lim et al., 1998).

Furthermore, BSG also acts via the PI3K-Akt signaling pathway. Treatment of fibroblast cells with recombinant BSG (rBSG) resulted in phosphorylation of Akt kinase and an increase in vascular endothelial growth factor (VEGF) production (Tang et al., 2006). Both the activation of Akt kinase and the induction of VEGF were specifically inhibited with a neutralizing antibody to BSG. The phosphoinositide 3-kinase (PI3K)-specific inhibitor LY294002 also inhibited VEGF production by BSG-overexpressing cells in a dose- and time-dependent manner (Tang et al., 2006).

BSG is also involved in the Wnt/β-catenin signaling pathway (Sidhu et al., 2010). Increasing BSG levels in lung tumor epithelial cells led to an increase in the metastatic potential of lung cancer cells through upregulation of Wnt/β-catenin signaling. Moreover, silencing of Bsg using shRNA significantly blocked this signaling pathway and inhibited cell migration, proliferation and tumor growth. Thus BSG utilizes several different intracellular signaling pathways that appear to be cell type and function specific.

**A.4 BSG and Tumor Cell Invasion**

Tumor cell invasion and metastasis are two important events in cancer biology and they are the most common causes of morbidity and mortality from cancer. The physical process of tumor invasion involves remodeling of the local microenvironment that
involves several factors. MMPs are critical mediators of proteolytic events during tumor invasion and play a central role in extracellular matrix (ECM) remodeling, inflammatory cell recruitment, cytokine activation, and regulation of angiogenesis (Donadio et al., 2008; Zheng et al., 2011).

BSG has been shown to stimulate the production of MMPs by tumor cells (Gabison et al., 2005) and BSG protein can be detected in conditioned medium from tumor cell cultures. Adding this conditioned medium to fibroblasts stimulates MMP production by fibroblasts but not vice versa (Ellis et al., 1989). Studies have shown that BSG stimulates human fibroblasts to produce MMP-1, MMP-2 and MMP-3 while an anti-BSG function-blocking antibody reduced MMP production (Hanata et al., 2007; Sato et al., 2009). BSG is also involved in regulation of expression of MMP-9 and MMP-11 in different types of cancer cells (Jia et al., 2007; Rucci et al., 2010). Moreover, BSG not only plays an important role in cancer progression by inducing MMP production, but also can stimulate tumor angiogenesis by regulating VEGF expression (Tang et al., 2005).

Studies have shown that BSG regulates tumor invasion both in vitro and in vivo. The expression of BSG is much stronger in colorectal high-grade adenoma, adenocarcinoma and metastatic carcinoma than in non-neoplastic superficial epithelium and low-grade adenoma (Zheng et al., 2011). Furthermore, human laryngeal carcinoma cells treated with either RNA interference approaches or an anti-BSG function-blocking antibody showed a decrease in invasion activity in vitro (Hanata et al., 2007; Zhu et al.,
2011). These findings indicate that BSG may be a good potential target for therapeutic anti-cancer drugs.

**A.5 BSG and Monocarboxylate Transporters (MCTs)**

Monocarboxylates such as lactate and pyruvate play an important role in cellular metabolism and metabolic communication between tissues. However, lactate cannot cross the plasma membrane by free diffusion. It requires a specific transport mechanism, provided by proton-linked monocarboxylate transporters (MCTs) (Halestrap and Price, 1999). Nine members of this MCT family have been identified based on sequence homologies. MCT1 and MCT4 have been shown to interact specifically with BSG (Kirk et al., 2000; Philp et al., 2003). Research studies demonstrated that BSG is involved in the translocation and correct localization of MCTs to the plasma membrane. Transfection of cells with MCT1 or MCT4 constructs alone resulted in only a few MCT molecules reaching the plasma membrane while most proteins were retained in the endoplasmic reticulum and Golgi apparatus. In contrast, when cells were co-transfected with both Bsg and MCT1 or MCT4 both proteins were expressed properly at the cell surface (Kirk et al., 2000). Moreover, in Bsg KO mice, there is a severe reduction in accumulation of the MCT1 and MCT3 proteins in the retinal pigment epithelium and a reduction in MCT1 and MCT4 in the neural retina. These results support a role for BSG in the targeting of these transporters to the plasma membrane (Philp et al., 2003).

**A.6 BSG and Integrins**

Integrins are members of a large family of cell adhesion molecules that are involved in
cell-extracellular matrix (ECM) and cell-cell interactions. Integrins are involved in a wide-range of biological functions including development, tissue repair, angiogenesis, inflammation and hemostasis (Keely et al., 1998). Two integrins, α3β1 and α6β1, have been found to associate with BSG at points of cell-cell contact (Berditchevski et al., 1997). Studies have shown that treating cancer cells with anti-α6β1-integrin antibodies significantly blocked the invasion-inducing capacity of BSG and secretion of MMPs (Dai et al., 2009).

A.7 Hormonal regulation of BSG in uterus

In mice, BSG immunostaining and mRNA were strongly localized in luminal and glandular epithelium on day 1 of pregnancy and gradually decreased to a basal level from day 2–4 of pregnancy. Bsg mRNA expression in the stromal cells is first detected on day 3 of pregnancy and increased on day 4 of pregnancy. On day 5 of pregnancy, the expression of both BSG protein and mRNA is only detected in the implanting embryos, and the luminal epithelium and stromal cells that surround the embryos. On day 6–8 of pregnancy, only a basal level of BSG protein was detected in the secondary decidual zone. Furthermore, estrogen significantly stimulated BSG expression in the ovariectomized mouse uterus. A high level of BSG immunostaining and mRNA was also seen in proestrus and estrus uteri (Xiao et al., 2002).

Similarly, BSG expression is also up regulated by estrogen in human uterine epithelium. During the proliferative phase, with high estrogen levels, BSG protein is strongly expressed in epithelial cells and only detected in the fibroblast cells that are near the
lumen. During the secretory phase of the cycle, progesterone levels are high and epithelial cells lose their expression of both progesterone and estrogen receptors. BSG expression is much weaker in glandular and luminal epithelial cells (Braundmeier et al., 2006).

A.8 BSG and Reproduction

Bsg gene knockout experiments have shown that Bsg mutant mice are sterile. This confirms that BSG plays a crucial role in both male and female reproduction (Igakura et al., 1998). In the Bsg knockout (KO) testes, there is a severe defect in spermatogenesis. Most of the spermatocytes in the Bsg KO mouse are arrested and degenerated at the metaphase of the first meiosis, and only a small number differentiate to step one spermatids. No mature sperm are found in the testis or the epididymis but the androgen levels in the mutant mice appear to be normal (Igakura et al., 1998). Similar phenotype has been also observed in the testes of Man2a2 KO mice (Akama et al., 2002), cAMP responsive element modulator (Crem) KO mice (Nantel et al., 1996) and Bcl-w KO mice (Print et al., 1998), which also show elevated apoptosis of male germ cells similar to Bsg KO males. Man2a2 is a gene that encoding α-mannosidase IIx (MX), an enzyme that forms intermediate asparagine-linked carbohydrates (N-glycans), results in Man2a2 null males that are largely infertile (Akama et al., 2002).

CREM is a nuclear target of the adenylyl cyclase pathway. It binds to cAMP response elements, modulates the transcription of cAMP responsive genes and regulates gene expression (Lalli et al., 1996). In human testes CREM expression is restricted to the
nuclei of round spermatids during spermatogenic stages I–III of seminiferous tubule differentiation. In the mouse, CREM is mainly expressed at stages VII-VIII and overall transcription stops at approximately stage IX (Sassone-Corsi, 1998; Weinbauer et al., 1998). The phenotype of the Crem KO mouse is very similar to the Bsg KO mouse (Nantel et al., 1996). For example, late spermatids are completely absent and there is a significant increase in apoptotic germ cells. BCL-W is a pro-survival protein belonging to the BCL2 family (Print et al., 1998). Bcl-w KO mice also show a marked reduction in the number of elongating spermatids. Moreover, no mature spermatozoa are found in the testes of Bcl-w KO mice. Understanding the mechanism of action of both CREM and BCL-W in the testis would definitely provide insights into the functions of BSG during spermatogenesis.

An in vitro RNA interference approach to inhibit BSG expression in germ cells (GC-1 and GC-2) showed that migration of the germ cells was reduced via reduced induction of MMP-2 production. This supports that BSG is involved in regulating migration of spermatogonia and spermatocytes during spermatogenesis (Chen et al., 2011). BSG is also required during fertilization. Fertilization of either zona pellucida-intact or zona pellucida-free oocytes was significantly inhibited in the presence of a monoclonal antibody to BSG (Saxena and Toshimori, 2004).

Several studies have shown that BSG also plays essential roles during implantation (Chen et al., 2007; Igakura et al., 1998). BSG is strongly expressed in the inner cell mass and trophectoderm of mouse blastocysts. Genotyping of embryos derived from
Bsg (+/-) parents showed that about 25% of embryos were Bsg KO at the blastocyst stage and there were no morphological differences between Bsg KO blastocysts and wild type (WT) blastocysts. However, only 7.8% Bsg KO embryos were found on day 8.5 of gestation. This observation indicates the majority of Bsg KO embryos failed to implant and died around the time of implantation and suggests that embryonic expression of Bsg is crucial during implantation. On the other hand, transfer of a total of 86 WT blastocysts into the uteri of Bsg KO female mice resulted in only 4 progeny (4.65%) while transfer of 90 WT blastocysts into WT female mice resulted in 41 progenies (45.45%). These results show that expression of Bsg in the maternal uterus also plays an important role during implantation. Taken together, these findings show that Bsg expression by both the embryo and the maternal uterus is essential for successful implantation.

B. Implantation

Implantation is the process by which the free floating blastocyst attaches to the uterine endometrium, invades into the stroma and establishes the placenta. It is the result of a complex series of reciprocal interactions between the conceptus and a receptive uterus. There are two main processes during implantation: uterine receptivity/conceptus invasion and the decidual cell reaction (decidualization) (Sharkey and Smith, 2003).

B.1 Uterine receptivity

Uterine receptivity is defined as a “window” of limited time when the uterine environment is conducive to support of conceptus attachment, growth, and the subsequent events of
implantation (Paria et al., 1993). The major regulators of uterine receptivity are estrogen and progesterone (P4). In the mouse, estrogen alone induces the proliferation of both luminal and glandular epithelial cells, whereas P4 is required for stromal cell proliferation (Huet-Hudson et al., 1989). On day 1 of pregnancy (vaginal plug), both luminal and glandular epithelial cells undergo proliferation in response to the preovulatory estrogen surge. In contrast, on day 3 of pregnancy, the rising level of P4 from the newly formed corpora lutea drives stromal cell proliferation which is then increased further by a small ovarian estrogen surge on day 4 of pregnancy (Huet-Hudson et al., 1989) (Fig. 2.2). Implantation is initiated in the dark phase between days 4 and 5 immediately after the blastocyst hatches from the zona pellucida (Finn and McLaren, 1967). Uterine sensitivity, which occurs in response to a series of hormonally dependent changes, is distinguished by pre-receptive, receptive and refractory (non-receptive) phases (Dey, 1996). In the mouse, the uterus is fully receptive to the conceptus on day 4 of pregnancy and the success rate of implantation decreases markedly after that point (Lim et al., 2002). This defined period of time that the uterus allows implantation to occur is commonly called the “implantation window” (Paria et al., 1993). On days 5-8 the trophoblast cells of the conceptus, originating from the trophectoderm, and endometrial decidual cells form a primitive diffusion barrier to allow nutrients and gases from the maternal blood vessels to nourish the conceptus (Jollie, 1990). At the same time as the receptive phase occurs, the uterine lumen has closed around the implanting blastocyst and an increased permeability of blood vessels is noted in the endometrium. This is followed by an intense edema in the endometrial zone surrounding the site of implantation. Because the permeability of blood vessels is
increased in the segments of the uterus containing an implanting blastocyst, an intravenous injection of blue dye which can bind to serum albumin provides a useful method for identifying very early implantation sites which appear as blue bands along the uterine horn (Finn and McLaren, 1967). The implantation sites are easily observable from approximately 24 hours and onward after the onset of implantation, as they enlarge. During the first 3-4 days after implantation is initiated this enlargement is mainly due to endometrial decidualization.

**B.2 Decidualization**

In the mouse, implantation triggers proliferation and then differentiation of endometrial stromal cells into large-polyploid decidual cells (decidua). This process is called the decidual cell reaction or decidualization. Stromal cell polyploidy during differentiation eventually results in apoptosis. By the end of the invasive period (day 11), the decidua has totally regressed. Decidualization provides the required environment for a conceptus to survive and develop until formation and differentiation of the placenta are completed approximately five days after the onset of implantation (Finn and McLaren, 1967). Decidual cells also serve an immunoregulatory role during pregnancy and regulate trophoblast invasion. Additional functions of the decidual cells are to support maternal blood vessel formation in order to perfuse and nourish the developing embryo (Ramathal et al., 2010). Decidualization first begins in the antimesometrial region of the uterus just adjacent to the conceptus and then spreads antimesometrially and laterally towards the myometrium. Approximately 4-6 cell layers of decidual cells are already present adjacent to the blastocyst when the luminal epithelium is displaced, and the
trophoblast cells reach the basal lamina in a small zone immediately surrounding the conceptus antimesometrially. This region is called the primary decidual zone (PDZ) and can be seen by Day 5. This PDZ is essentially avascular and is believed to act as a barrier to the passage of macromolecules between the conceptus and maternal blood. The PDZ is believed to protect the embryo against exposure to microorganisms, maternal IgG, and immunocompetent cells; providing a favorable environment for the survival of the embryo during early implantation (Parr et al., 1986). Starting on Day 6, a broad, well-vascularized zone is visualized beginning from the PDZ and extending antimesometrially and laterally towards the myometrial region. This is called the secondary decidual zone (SDZ). Together, the PDZ and SDZ make up the antimesometrial decidua. The majority of the cells in the antimesometrial decidua are transient tissue, as most of these cells undergo apoptosis (Gu et al., 1994; Rogers et al., 1983). The last region of the uterus where decidualization occurs is in the mesometrial region of the endometrium. These mesometrial decidual cells can be seen on day 7 and show slight morphological differences from antimesometrial decidual cells. This mesometrial region is where the trophoblast giant cells (TGC) invade and the placenta forms (Welsh and Enders, 1993). In the mouse there are several well-known markers of decidualization such as alkaline phosphatase (ALP) and decidual prolactin-related protein (PRP) (Li et al., 2007).

Decidualization can be induced artificially through a mechanical stimulus in the absence of embryos. There are several well-established artificial methods to induce decidualization either in vivo or in vitro (Lundkvist and Nilsson, 1982; Ryan et al., 1994;
Tang et al., 1993). In order to distinguish the resulting tissue from that of the uterus of pregnancy, the artificially induced tissues are called deciduoma. These experimental methods provide a useful way to study the endometrial changes that occur during decidualization without the potential confounding influences from the conceptus.

Unlike rodents, the decidualization in human is a conceptus-independent process. Decidualization for pregnancy is initiated by tissue remodeling during the secretory phase of each menstrual cycle following ovulation. Similar to mouse, it is also initiated by the presence of P4 after proper E2 priming. This event first begins in the late secretory phase in endometrial stromal cells surrounding the spiral arteries of the uterus and these transform into enlarged round cells with specific ultrastructural modifications, accompanied by the secretion of specific markers such as prolactin (PRL) and IGF binding protein-1 (IGFBP-1) (Garrido-Gomez et al., 2011; Ramathal et al., 2010). Human endometrial stromal cells can also be induced to decidualize artificially in vitro. Decidualization in vitro requires E2 and P4 as well as the additional presence of an exogenous stimulator (cAMP). The process of in vitro decidualization in human stromal cells is several days longer than that in mouse stromal cells (Li et al., 2007).

**B.3 Molecular signaling during implantation**

Almost all our knowledge of molecular signaling during implantation is derived from rodent models. Gene knockout techniques have provided us with a very powerful approach to identify specific genes that play essential roles.
In mice, the cytokine leukemia inhibitory factor (LIF) is a maternal product that is absolutely required in the uterus for proper embryo implantation (Stewart et al., 1992). LIF is expressed at low levels in the nonpregnant uterus, but at the time of implantation, LIF is highly expressed in the glandular epithelium by midday on day 4 of pregnancy. Gene knockout experiments indicate that females lacking a functional LIF gene are fertile, but their blastocysts fail to implant and do not develop. LIF is up-regulated by Hmx3 and acts on cells by binding to the heterodimeric LIF receptor consisting of the two transmembrane proteins, gp130 and the LIF receptor α (LIFRα). Deletion of Hmx3 or a mutant gp130 deficient in STAT3 signaling, all result in the failure of implantation (Cheng et al., 2001; Ernst et al., 2001; Sharkey and Smith, 2003; Wang et al., 1998). Following the peak of LIF expression, around 1800 hours on day 4 of pregnancy, the heparin-binding EGF-like growth factor (HB-EGF) gene is expressed in the mouse uterine luminal epithelium surrounding the blastocyst 6-7 hours before embryo attachment (Das et al., 1994). HB-EGF is the earliest known marker of implantation in mice, because it is expressed in the uterine luminal epithelium surrounding the pre-attachment blastocyst (Paria et al., 2002). In addition, it has been shown that beads soaked in HB-EGF induce many of the same discrete local responses in the endometrium elicited by the blastocyst, including increased localized vascular permeability, decidualization, and expression of Bmp2 at the sites of the beads (Paria et al., 2001). One hallmark of implantation is increased vascular permeability at the implantation site by 2100 hours on day 4 of pregnancy. COX-2 is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) which are key mediators of increased endometrial vascular permeability during implantation and decidualization.
COX-2 is expressed in the luminal epithelium and underlying stromal cells solely at the sites of blastocyst attachment. Targeted disruption of COX-2 in mice produces multiple failures in female reproductive processes that include ovulation, fertilization, implantation, and decidualization (Lim et al., 1997). COX-2 down-regulation is also found in Homeobox A10 (Hoxa 10) null uteri resulting in impaired decidualization and rendering these uteri non-receptive to embryo implantation (Ramathal et al., 2010).

The zone of decidualization spreads out from the implantation site over the next 2 days and a successful reaction requires the action of numerous genes such as interleukin-11, interleukin-11 receptor α (IL-11Rα), Bone Morphogenetic Protein-2 (BMP2) and Wingless 4 (Wnt4) etc. IL-11 is highly expressed in the decidua upon embryo implantation, and its receptor, IL-11Rα, is also localized in decidua. IL-11Rα null mice display an impaired decidual response and inhibition of IL-11 function with an antagonist disrupts the proliferation and differentiation of stromal cells in pregnant mice (Bilinski et al., 1998; Menkhorst et al., 2009; Robb et al., 1998). BMP2 is expressed in the stromal cells surrounding the implanted embryo and is essential for a successful decidual response. BMP2-null mice are infertile due to the absence of a decidual response. Wnt4 is a downstream target of BMP2 in stromal cells undergoing decidualization (Li et al., 2007). Wnt4 is expressed in stromal cells surrounding the blastocyst at the onset of implantation followed by its expression in the decidua with the progression of pregnancy. Ablation of Wnt4 renders female mice subfertile due to a defect in embryo implantation and subsequent defects in endometrial stromal cell survival, differentiation, and responsiveness to progesterone signaling (Franco et al., 2011; Paria et al., 2001).
C. Spermatogenesis

Spermatogenesis is the process of producing sperm (haploid) from initially undifferentiated germ cells (diploid). In mammals, spermatogenesis occurs within the seminiferous tubule and can be divided into three distinct phases: spermatogonial renewal and proliferation, meiosis, and spermiogenesis. In the first phase of spermatogenesis, spermatogonial stem cells proliferate into type A spermatogonia, intermediate spermatogonia and type B spermatogonia. Type B spermatogonia then proliferate to give rise to primary spermatocytes. Spermatogonial stem cells must be able to maintain their number by self-renewal. The second phase of spermatogenesis involves reduction of chromosome number through meiosis in primary spermatocytes. After the first meiotic division secondary spermatocytes quickly enter the second meiotic division to form the haploid spermatids. During the third phase, spermiogenesis, these haploid spermatids undergo a complex series of cytological transformations leading to the production of the mature spermatozoa (Bellve et al., 1977).

During spermatogenesis, there are a group of somatic cells named Sertoli cells that have direct contact with all the different types of germ cells in the seminiferous tubules. Sertoli cells play a major role in the regulation of germ cell differentiation and migration. First, these cells form the blood-testis barrier in the seminiferous epithelium near the basement membrane (Dym and Fawcett, 1970). This immunological barrier protects the postmeiotic germ cells, including sperm, from the host immune system to avoid attack from the production of antibodies against germ cell specific antigens (Wong et al., 2010). Sertoli cells are also responsible for the formation and secretion of seminiferous
tubule fluid (STF) which consists of a number of products that are involved in spermatogenesis such as transport and binding proteins, proteases and protease inhibitors, hormones and growth factors (Russell and Griswold, 1993; Skinner and Griswold, 2005). STF is important for maintaining the nutritional and hormonal microenvironment necessary for the viability and differentiation of germ cells during spermatogenesis. It also provides the milieu for developing spermatozoa and the vehicle to transport them from the testis (Rato et al., 2010; Richburg et al., 1994). Furthermore, there is strong evidence that some ligands secreted by Sertoli cells can modulate gene expression in germ cells during spermatogenesis (Tsuruta and O'Brien, 1995). Sertoli cells communicate with germ cells through direct cell-cell contact as well as paracrine interactions. Loss of these interactions between Sertoli cells and germ cells leads to the failure of spermatogenesis. For example, N-acetylglucosamine (GlcNAc) terminated tri-antennary and fucosylated N-glycan structures have been shown to play a key role in germ cell adhesion to Sertoli cells. A 50% reduction in expression of these N-glycans in the testis led to almost complete suppression of fertility (Akama et al., 2002).

D. Biosynthesis of N-glycan Carbohydrates in Mammalian Cells

Glycosylation has been classified as one of two types, either N-glycosylation or O-glycosylation. O-glycans are relatively short sugar chains linked to hydroxylated amino acids of proteins (serine or threonine). O-glycans are involved in a number of functions including inflammatory responses and lymphocyte homing (Fukuda and Akama, 2003; Saez et al., 1999). N-glycans are branched carbohydrates attached to the amide
nitrogen of asparagines residues of peptides. There are three broad classes of N-glycans: high mannose, hybrid, and complex and all are found attached to mammalian glycoproteins (Alberts, 2002; Fukuda and Akama, 2003). They all share a common core structure which contains two N-acetylglucosamines (GlcNac) and three mannoses, but differ in their outer branches. The high mannose type N-glycans typically have two to six additional mannose residues and the complex type structure contains two to five outer branches with the addition of further sugars. The reason some types of N-glycans are classified as hybrid is because they have features of both the high mannose and complex types of N-glycans (Kornfeld and Kornfeld, 1985) (Fig. 2.3).

Biosynthesis of N-glycans includes “en bloc” transfer of oligosaccharides made of glucose, mannose and N-acetylglucosamine (GlcNac) utilizing several different enzymes to complete the process. For example, N-acetylglucosaminyltransferase I (GlcNAc-TI) is essential for the conversion of high mannose type N-glycans to hybrid type N-glycans. Mice lacking GlcNAc-TI die at embryonic day 10 due to being developmentally delayed (Ioffe and Stanley, 1994). N-acetylglucosaminyltransferase II (GlcNAc-TII) is a glycosyltransferase which is essential for the synthesis of complex type N-glycans in the Golgi. GlcNAc-TII null mice have severe neuronal, osteogenic, hematopoietic and gastrointestinal problems (Wang et al., 2001). Another important enzyme is MX which removes two mannosyl residues from high mannose type N-glycans (Akama and Fukuda, 2006). Mice lacking MX show a great reduction in the level of GlcNAc-terminated N-glycans, and the spermatogenic cells of MX null mice fail to adhere to Sertoli cells which leads to failure of spermatogenesis.
Fig. 2.1. The structure of basigin. BSG is a transmembrane glycoprotein and belongs to the immunoglobulin superfamily. Four conserved cysteine residues in the extracellular domain form two disulfide bridges. Three potential N-glycosylation sites are represented by symbol (о— ).
Fig. 2.2. Window of receptivity in the mouse. On day 1 of pregnancy, ovarian estrogen released at ovulation induces luminal and glandular epithelial cell proliferation. On day 3, the rising level of progesterone from newly formed corpora lutea shifts the wave of proliferation from epithelial cells to stromal cells. A small estrogen surge on day 4 is required to increase stromal cell proliferation in preparation for implantation. The mouse uterus is fully receptive to the conceptus on day 4 of pregnancy.
Fig. 2.3. Structure of the three major types of N-glycans. The boxed area represents the common core region for all the N-linked structures. It typically contains two GlcNac and three mannoses.
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Chapter Three

BASIGIN is Essential for Sensitizing the Uterine Luminal Epithelium in Mice During Early Pregnancy

Abstract

Basigin (BSG) is a multifunctional glycoprotein that plays an important role in female reproduction since it has been shown that female knockout (KO) mice are infertile. The aim of the present study was to determine 1) whether BSG expression in the uterus is dependent on estrogen receptor-α (ESR1, ERα) or -β (ESR2, ERβ); and 2) whether BSG is required for proliferation of the uterine luminal epithelium during early pregnancy in preparation for implantation. Expression of BSG protein in the female reproductive tract was studied in Esr1-null (αERKO), and Esr2-null (βERKO) mice by immunohistochemistry and immunoblotting. Bsg mRNA levels in the ovary and uterus were examined by quantitative RT-PCR (qPCR). BSG protein was expressed in the uterine epithelium at estrus in βERKO mice but not in αERKO mice. However, a higher level of Bsg mRNA was observed in the uteri of αERKO mice as compared with wild type (WT) and βERKO mice. In the mouse, estrogen alone induces the proliferation of both luminal and glandular epithelial cells during early pregnancy. On day 1 of pregnancy, the expression levels of ERα and a well-known estrogen responsive gene, MUC1, appeared to be normal in the uteri of Bsg KO females. This suggested that the circulating estradiol levels in Bsg KO mice are normal. I examined proliferation in uterine epithelial cells and found that, in WT mice, uterine epithelial cells were highly proliferative as measured by expression of Ki67 in both the luminal and glandular epithelial cells. In contrast, Ki67 expression was significantly decreased in the epithelial
cells of *Bsg* KO uteri. Immunochemistry using the BSG antibody revealed that the expression of BSG in C/EBPβ null uteri was heterogeneous and spotty in the luminal epithelium as compared with a more homogeneous expression pattern of BSG in the uterine epithelium of WT uteri. These results indicate that C/EBPβ may be one of the factors regulating the expression of BSG in mouse uterine epithelium. My findings suggest that expression of BSG protein in the uterus requires estrogen acting through ESR1, but not ESR2. Moreover, estrogen may regulate the proliferation of both luminal and glandular epithelial cells in the uterus during early pregnancy through a pathway involving BSG.

**Introduction**

Implantation is the process by which the conceptus comes into intimate contact with the endometrium so that the mother can provide nutrients to the developing fetus. It begins with the apposition of the blastocyst to the luminal epithelium of the endometrium and culminates in the formation of the definitive placenta (Bany and Cross, 2006). There are two main processes during implantation in the rodent: uterine receptivity/conceptus invasion and the decidual cell reaction (decidualization) (Sharkey and Smith, 2003).

Uterine receptivity is defined as a “window” of limited time when the uterine environment is conducive to support of conceptus attachment, growth, and the subsequent events of implantation (Paria et al., 1993). The major regulators of uterine receptivity are estrogen and progesterone (P4). In the mouse, estrogen alone induces the proliferation of both luminal and glandular epithelial cells, whereas P4 is required for stromal cell
proliferation (Huet-Hudson et al., 1989). On day 1 of pregnancy (vaginal plug), both luminal and glandular epithelial cells undergo proliferation in response to the preovulatory estrogen surge.

BSG is abundant in the reproductive organs and plays essential roles in both male and female reproduction (Chang et al., 2004; Igakura et al., 1998; Kuno et al., 1998). Several studies have shown that Bsg plays essential roles during implantation (Chen et al., 2007; Igakura et al., 1998). Bsg is strongly expressed in the inner cell mass and trophectoderm of mouse blastocysts. Genotyping of embryos derived from Bsg (+/-) parents showed that about 25% of embryos were Bsg knockout (KO) at the blastocyst stage and there were no morphological differences between Bsg KO blastocysts and wild type (WT) blastocysts. However, only 7.8% Bsg KO embryos were found on day 8.5 of gestation. This observation indicates the majority of Bsg KO embryos failed to implant and died around the time of implantation and suggests that embryonic expression of Bsg is crucial during implantation. On the other hand, embryo transfer studies showed that transfer of 86 WT blastocysts into the uteri of Bsg KO female mice resulted in only 4 progeny (4.65%) whereas the transfer of 90 WT blastocysts into WT female mice resulted in 41 progenies (45.45%). These results show that expression of Bsg in the maternal uterus also plays a critical role during implantation. Taken together, these findings show that Bsg expression by both the embryo and the maternal uterus is essential for successful implantation. Furthermore, previous studies have shown that decidualization occurs 24 hours earlier in Bsg KO mice as compared to WT mice (Chen, unpublished observations). Treatment of mouse stromal cells with rBSG significantly
suppressed stromal cell decidualization (Chen et al., 2009). Thus it seems that BSG may play a role in regulating the timing of decidualization in mice.

The steroid hormone, estradiol, acting via the nuclear receptors, estrogen receptor-α (ESR1, ERα) and -β (ESR2, ERβ), is fundamental to the regulation of numerous genes in reproductive tissues. Both luminal and glandular epithelial cells undergo proliferation on day 1 of pregnancy in response to the preovulatory surge of estradiol. This preovulatory increase in estradiol also sensitizes the uterine epithelium for attachment and subsequent implantation of the blastocyst. BSG protein and mRNA expression are strongly localized in the luminal and glandular epithelium on day 1 of pregnancy and expression is up regulated by estrogen (Xiao et al., 2002). However, the role of BSG during early pregnancy and the role of ESR1 and ESR2 in the regulation of BSG expression in the uterus remain unknown. The goals of the current study were 1) to compare the expression of BSG in the reproductive tissues of WT, Esr1 null (αERKO), and Esr2 null (βERKO) mice to determine the contribution of each estrogen receptor isoform to BSG expression; and 2) to investigate the importance of BSG for uterine epithelial cell proliferation during early pregnancy.

Materials and Methods

Animals

Animals used in this research were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Illinois. The wild-type C57BL6/J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and
were housed under temperature and light controlled conditions (12 h light: 12 h dark) with free access to food and water. C57BL6/J heterozygous (Bsg +/-) mice were a kind gift from Dr. Takashi Muramatsu, Department of Biochemistry, Nagoya University School of Medicine, Japan. Heterozygote breeding was carried out in the animal facility of the University of Illinois. At 3 weeks of age, offspring were anesthetized by isoflurane inhalation (Attane; Minrad, Bethlehem, PA) and tail snips were collected for genotyping. DNA was extracted from the snips using REDExtract-N-Amp (Sigma, St. Louis, MO), and genotypes determined by PCR using primers for Bsg and neomycin (Igakura et al., 1998). Offspring with the Bsg -/- phenotype were housed under the same conditions as those for the WT mice. Female WT and Bsg-null mice were ovariectomized at 10 to 12 weeks of age and rested for 2 weeks. These mice were then treated with a single dose of 17β-estradiol (E) (200 ng in sesame oil) by intraperitoneal (i.p.) injection and uteri were harvested at 24 hours after injection.

The generation of mice null for the Esr1 (ERα) or Esr2 (ERβ) genes was in a C57BL6/J background and has been described previously (Krege et al., 1998; Lubahn et al., 1993). All mice used for this study were 40-60-days old and provided by Dr. Kenneth S. Korach, Laboratory of Reproductive and Developmental Toxicology, National Institutes of Health, Research Triangle Park, NC.

Histological sections of uteri from C/EBPβ null mice were provided by Dr. Milan Bagchi of University of Illinois. Briefly, female WT and C/EBPβ-null mice of the 129Sv background were ovariectomized at 10 to 11 weeks of age and rested for 2 weeks.
These mice were then treated with a single dose of 17β-estradiol (E) (250 ng in sesame oil) by intraperitoneal (i.p.) injection and uteri were harvested at various times after injection.

**Tissue collection**
All mice were euthanized with carbon dioxide. Uteri and ovaries were collected immediately. Tissues for histology were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS overnight and processed for paraffin embedding. Tissues for immunoblotting analyses were snap-frozen in liquid nitrogen and stored at -80°C until use.

**Immunoblotting**
Frozen whole uteri isolated from WT and αERKO mice were ground in liquid nitrogen and solubilized in modified RIPA buffer in the presence of protease inhibitors. Protein concentrations were measured by BCA protein assay (Fisher Scientific, Pittsburgh, PA). Twenty micrograms of total protein were resolved by 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes (PVDF) (Millipore, Billerica, MA), blocked in 5% non-fat dry milk, and probed with 0.2µg/ml goat polyclonal antibody against mouse BSG (R&D, Minneapolis, MN) overnight at 4°C. The membranes were washed and incubated with the HRP-conjugated donkey anti-goat IgG antibody (Santa Cruz Company, Santa Cruz, CA) at 1:3000 dilution for 60 min at room temperature. The bound secondary antibody was detected using SuperSignal West Pico Chemiluminescent Substrate
(Fisher Scientific). The same membranes were stripped and reprobed with anti-GAPDH antibody (Cell Signaling, Boston, MA) as a loading control.

**Densitometric analysis of immunoblotting**

Films developed after exposure to chemiluminescence were scanned, and images were analyzed using the ImageJ software from NIH (available at http://rsbweb.nih.gov/ij/download.html). Measurements of BSG bands were normalized for loading differences using measurements of GAPDH. Graphs represent means and SEM.

**RNA isolation and quantitative reverse transcription-PCR (qPCR)**

Total RNA was extracted from mouse uteri and ovaries using TRIzol™ (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies; Carlsbad, CA) following the manufacture’s instructions. qPCR analyses were performed using TaqMan® Universal PCR Master Mix No AmpErase® UNG (Life Technologies; Carlsbad, CA). Genes were amplified using 20 x Assays-on-Demand™ Gene Expression Assays purchased from Life Technologies. The following 20x Assays-on-Demand™ Gene Expression Assay primer-probe sets were used for my studies: *Bsg* (Mm00814798_m1), Lactoferrin (*Ltf*) (Hs00236877_m1), and 18S (Hs99999901_s1). Briefly, 4.5 µl of a 1:10 diluted cDNA sample was mixed with 5.5 µl of master mix (5µl of TaqMan Universal PCR Mix and 0.5 µl of 20X Assay on Demand) for a total volume of 10 µl per well in a MicroAmp optical
384-well reaction plate. Three experimental replicates were performed for each sample. qPCR amplification and quantitation was performed using the ABI 7900 sequence detection system for 40 cycles (95°C for 15 sec, 60°C for 1 min). The comparative CT method (ΔΔCt) was used for quantification of gene expression. Relative fold differences in gene expression for all tested genes were normalized to the 18S endogenous control.

**Immunohistochemistry**

Paraffin embedded tissues were sectioned at 5 µm and mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, sections for immunostaining were boiled in 10 mM citrate buffer (Mix 18 ml of 0.1 M citric acid, 82 ml of sodium citrate and 900 ml of H₂O, pH 6.0) for 30 minutes to promote antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide for 15 minutes. After blocking with 2% normal serum (Vector Laboratories, Burlingame, CA) in PBS/Tween 20 (PBS/T) for 60 minutes, sections were incubated at 4°C overnight with: 2 µg/ml anti-BSG (R&D, Minneapolis, MN, USA), anti-Ki67, 1:50 (Dako, Carpinteria, CA), anti-ESR1, 1:200 (Santa Cruz, Santa Cruz, CA), and anti-MUC1, 1:100 (Novus Biologicals, Littleton, CO). Isotype control IgG was applied as a negative control. After washing in PBS/T, sections were incubated with secondary antibody conjugated with biotin (Vector Laboratories) diluted at 1:200 with PBS/T for 60 minutes at room temperature. Sections were then incubated in ABC solution (Vector Laboratories) for 30 minutes, reacted with metal-3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO) for minutes, and counterstained with hematoxylin.
Statistical analysis

Results are presented as the mean ± SEM of at least three independent samples. Statistical analyses were performed by ANOVA. Statistical significance was set as \( P<0.05 \).

Results

BSG protein and mRNA levels in uteri of WT, αERKO, and βERKO mice

I first examined expression of BSG protein levels in the uteri of αERKO and βERKO mice. BSG immunostaining fluctuated throughout the estrous cycle in the uterus of βERKO mice (Fig. 3.1C). In contrast, luminal and glandular epithelial cells showed very weak staining for BSG at stage of diestrus transition to proestrus (Fig. 3.2D). However, BSG expression was completely absent in the luminal epithelium of αERKO mice (Fig. 3.1A) whereas BSG was detectable in myometrium (B). There was no staining in IgG negative controls (Fig. 3.1D).

BSG protein levels in uteri of αERKO mice were markedly reduced to around 40% of those of WT uteri (Fig. 3.2A and B). Although my immunoblotting results suggested that ESR1 is required for normal BSG protein expression in the uterus, it was not clear whether this is a transcriptional effect on Bsg mRNA levels. To answer this question, I measured Bsg mRNA levels in the uteri of αERKO and βERKO mice. Since lactoferrin is also an estrogen-regulated gene whose expression is mediated through ESR1, I used lactoferrin as a control gene. As expected, lactoferrin mRNA levels in the uteri of αERKO mice were only 20% of those of WT mice (Fig. 3.3C). However, in contrast to
the lack of BSG protein expression in the uterine epithelium of αERKO mice and the overall reduced levels of BSG protein in the uteri. Bsg mRNA levels in uteri of αERKO mice were threefold increased compared with those of WT mice (Fig. 3.3A). Bsg mRNA levels in uteri of βERKO mice and ovaries of αERKO and βERKO mice were comparable to those of WT mice (Fig. 3.3A and B).

**Proliferation in the uterine epithelium at day 1 of pregnancy in WT and Bsg KO mice**

Expression of Ki67, a proliferation marker, was determined in the uteri of WT and Bsg KO mice at day 1 of pregnancy. Immunochemistry with a Ki67 antibody revealed that Ki67 was highly expressed in both the luminal and glandular epithelial cells of WT mice (Fig. 3.4A). In contrast, Ki67 expression was significantly decreased in the epithelial cells of Bsg KO uteri (Fig. 3.4B). In addition, the size of the uteri of WT mice was much larger than that of the Bsg KO mice (Fig. 3.4A, B).

**Expression of ESR1 and MUC1, an estrogen-regulated marker, on day 1 of pregnancy in the uteri of WT and Bsg KO mice**

Since proliferation of uterine epithelial cells is highly estrogen dependent, it was important to determine whether the expression of ESR1 was altered in the Bsg KO uterus at this time. MUC1 is a transmembrane mucin expressed on the apical surface of uterine epithelial cells. It lubricates and hydrates cell surfaces and functions as a protective barrier against microbial and enzymatic attack (Dharmaraj et al., 2009). MUC1 is up regulated by estrogen in the mouse (Surveyor et al., 1995) and is well
accepted as a marker for estrogen responsiveness. I analyzed expression of MUC1 on
day 1 of pregnancy in the uteri of both WT and Bsg KO mice. My results showed that
ER\(\alpha\) was highly expressed in the luminal epithelium of both WT (Fig. 3.5A) and Bsg KO
mice (Fig. 3.5B). Moreover, MUC1 was also highly expressed at the apical surface of
uterine epithelia in both the WT and Bsg KO uteri (Fig. 3.5C and D). This suggested
that the markedly reduced proliferation of uterine epithelial cells in the Bsg KO females
is not due to a loss in the ability to respond to estrogen.

**Ki67 expression in the uteri of ovariectomized WT and Bsg KO mice treated with
200ng estradiol.**

I next investigated whether ovariectomized Bsg KO mice could respond normally to an
injection of exogenous estradiol in terms of uterine cell proliferation. Immunochemistry
with the Ki67 antibody revealed that Ki67 was highly expressed in the luminal epithelial
cells of both WT mice (Fig. 3.6A) and the Bsg KO mice (Fig. 3.6B). This was a
surprising finding and suggests that the luminal epithelial cells of the Bsg KO mice are
able to respond to exogenously administered estradiol with a normal proliferative
response but not to the endogenous levels of estradiol present at day 1 of pregnancy.

**BSG immunoreactivity in the uteri of ovariectomized WT and C/EBP\(\beta\) KO mice
treated with 250ng estradiol.**

CCAAT enhancer binding protein beta (C/EBP\(\beta\)) is an estrogen-regulated transcription
factor expressed in female reproductive tissues that is essential for uterine functions
(Mantena et al., 2006). Immunochemistry with the BSG antibody revealed that the
expression of BSG in C/EBPβ null uteri was heterogeneous and spotty in the luminal epithelium (Fig. 3.7B, D). In contrast, BSG was highly expressed homogeneously in the uterine epithelium of WT mice (Fig. 3.7A, C). There was no immunostaining in the IgG control sections (Fig. 3.7E).

Discussion

BSG is a transmembrane glycoprotein that is expressed in several reproductive tissues and plays an essential role in both male and female fertility (Igakura et al., 1998; Kuno et al., 1998). Previous studies have shown that BSG expression is up regulated by estrogen (Xiao et al., 2002). Estrogen significantly induced both BSG protein and mRNA expression in the luminal and glandular epithelium of ovariectomized mice. Both BSG mRNA and protein expression were also seen in the same localization in the estrous uterus, during which maternal estrogen was at a high level. However, the role of ESR1 and ESR2 in the regulation of BSG expression in uterus was unknown. By comparing the level and pattern of BSG expression in the uteri of αERKO, and βERKO mice, I determined that BSG expression in the βERKO uterine endometrium was localized in the luminal and glandular epithelium and clearly fluctuated during the cycle such that the expression was markedly high at estrus when estrogen levels are high, similar to previous studies in WT mice. In contrast, BSG expression was completely absent in the luminal epithelium of αERKO mice. These results indicated that estrogen regulation of BSG expression in the uterine epithelium occurs via ESR1. Surprisingly, I observed a significant increase, instead of a decrease, in Bsg mRNA levels in the uteri of Esr1-null females. These apparently contradictory observations suggest that the signaling
mediated by ESR1 must be necessary for BSG protein translation, rather than mRNA transcription. Knockout of Esr1 results in a lower BSG protein expression in the uteri, which may lead to a compensatory response with increases in Bsg mRNA levels.

On day 1 of pregnancy, both luminal and glandular epithelial cells undergo proliferation in response to the preovulatory surge of estradiol (Huet-Hudson et al., 1989). This preovulatory increase in estradiol also sensitizes the uterine epithelium for attachment and subsequent implantation of the blastocyst. Bsg is essential for successful implantation as very few, if any, embryos are able to implant in the uterus of Bsg KO females (Igakura et al., 1998). However, it is not known how the loss of Bsg expression might alter the uterine epithelium of female Bsg KO mice. Ki67 is a nuclear protein that is expressed in the G1, S, G2, and M phases of the cell cycle and is considered a reliable marker of cell proliferation (Barnard et al., 1987; Gerdes et al., 1983). In the present study, I found that Ki67 expression was significantly decreased in the epithelial cells of Bsg KO uteri, while ERα and MUC1, an estrogen responsive gene, were normally expressed in the luminal epithelium of both WT and Bsg KO mice. BSG is highly expressed in proliferative cells (Biswas and Nugent, 1987). For example, BSG is expressed most strongly in both luminal and glandular epithelial cells at estrus (Chen et al., 2010; Xiao et al., 2002). BSG is also highly expressed in basal cells of the epidermis and expression decreases as the cells differentiate. Coincidentally, the basal cells are the only highly proliferative cells within normal human epidermis (Chen et al., 2001). Moreover, several studies have shown that malignant, low differentiated tumor cells express higher levels of BSG compared with their normal or benign counterparts.
(Polette et al., 1997; Sameshima et al., 2000). All of these results suggest that BSG is abundant in proliferative and undifferentiated cells. BSG also regulates the proliferation of several different cells including human stromal cells (chapter three Fig. 4.1) and spermatocytes (unpublished data). Taken together, my results suggest that estrogen alone may regulate the proliferation of both luminal and glandular epithelial cells through a pathway involving BSG during early pregnancy. To further validate the effect of estrogen on the expression of BSG, I tested the effects of exogenously administered estradiol (200ng) on uterine epithelial cell proliferation in ovariectomized WT and Bsg KO mice. Surprisingly, I observed a very similar pattern of expression of Ki67 in both the WT and Bsg KO uteri. I hypothesize that Bsg is necessary for normal uterine epithelial cell proliferation in response to estradiol but probably involves interactions with some other signaling pathway.

The results of my current study showed that Ki67 expression was significantly decreased in the epithelial cells of Bsg KO uteri. A similar phenotype has been observed in C/EBPβ null uteri (Mantena et al., 2006). The transcriptional factor C/EBPβ is an estrogen-regulated gene in female reproductive tissues that is essential for uterine functions (Mantena et al., 2006). Previous studies have shown that female mice lacking the transcription factor C/EBPβ are infertile and display markedly reduced estrogen-induced proliferation of the uterine epithelial cells during the reproductive cycle (Ramathal et al., 2010). Administration of estrogen elicited only a weak increase in immunostaining for Ki67 in the uterine epithelium of the C/EBPβ KO mice, indicating a poor proliferative response in the absence of this transcription factor. In contrast, the
expression of ERα and lactoferrin, a well characterized estrogen–ESR1 complex regulated gene, (Stokes et al., 2004; Zhang and Teng, 2000) remained unaltered in the C/EBPβ-null epithelium. Loss of C/EBPβ leads to a significant impairment in DNA replication in uterine luminal epithelial cells. The expression and cellular localization of cyclin E, E2F3, and p27 are markedly reduced. This proliferation defect in C/EBPβ-null uterine luminal epithelial cells is also associated with the activation of well-known DNA damage response pathways involving ataxia telangiectasia mutated (ATM), ATM-Rad3 related (ATR), the checkpoint kinases Chk1 and Chk2, and p53. Activated p53 helps to maintain the G1-S-phase blockade of the cell cycle by inducing the synthesis of the inhibitor p21 (Ramathal et al., 2010). Immunochemistry using the BSG antibody revealed that the expression of BSG in C/EBPβ null uteri was heterogeneous and spotty in the luminal epithelium as compared with a more homogeneous expression pattern of BSG in the uterine epithelium of WT uteri. These results indicate that the expression of BSG in mouse uterine epithelium is not completely dependent on the presence of C/EBPβ. C/EBPβ may not be the only factor regulating the expression/localization of BSG in mouse uterine epithelium. In other words, C/EBPβ may not regulate the expression of BSG directly or may only be involved in estrogen-stimulated expression of BSG. A better understanding of the mechanism of action of C/EBPβ in the uterine luminal epithelium would definitely provide further insights into the regulation and functions of BSG during early pregnancy.

In summary, I have used estrogen receptor-null mice to demonstrate that BSG expression is estrogen and ESR1-dependent in uterine epithelial cells. BSG may play a
role in sensitizing the luminal epithelium during early pregnancy. Future studies will focus on identifying additional regulators of BSG expression in luminal epithelium during early pregnancy.
Figure 3.1. BSG expression in uteri of αERKO (A, B) and βERKO (C, D, E) mice (n=3). Uteri were collected at estrus (C) and diestrus transitions to proestrus (D) from βERKO mice. Sections were stained with goat anti-mouse BSG antibody (A, B, C and D) or goat non-specific IgG (E) as a negative control. BSG immunostaining was intense in both luminal and glandular epithelium of βERKO mice at estrus, weaker at the stage of diestrus transitions to proestrus, and was not observed in the luminal epithelium of αERKO uteri (A) whereas BSG immunoreactivity is detectable in myometrium (arrow heads in B). The brown color represents the localization of BSG protein and there was no staining evident in the negative controls. Scale bar, 20 µm
Figure 3.2. BSG protein levels in uteri of WT (n=4) and ERKO mice (n=3). (A) Uterine proteins were isolated from whole uteri and subjected to immunoblotting. (B) Bar graphs summarizing the densitometric analysis of the immunoblotting. BSG protein levels of αERKO uteri were 40% of those of WT mice. Bars represent the mean ± SEM of at least three independent mice with the data normalized to the loading control, GAPDH. The asterisk indicates a statistical difference (P<0.05) compared with WT tissues.
Figure 3.3. *Bsg* mRNA levels in uteri of WT (n=4) and ERKO mice (n=4). Uterine RNA was isolated from whole uteri and subjected to qPCR analyses. (A) *Bsg* mRNA levels in uteri of αERKO and βERKO mice were comparable to those of WT mice. (B) *Bsg* mRNA levels in ovaries of αERKO and βERKO mice were comparable to those of WT mice.
Figure 3.3 (cont’d). (C) Lactoferrin mRNA levels in uteri of αERKO mice were comparable to those of WT mice. The data are presented as fold changes relative to tissues of WT mice, and data are mean ± SEM. The asterisk indicates a statistical difference (P<0.05) compared with WT tissues.
Figure 3.4. Ki67 immunoreactivity in the uteri of WT and Bsg KO mice on day 1 of pregnancy (n=3). (A) Localization of Ki67 protein in the uteri of WT mice. (B) Localization of Ki67 protein in uteri of Bsg KO mice. The rectangles located at the bottom of the images are the enlarged views of the highlighted areas in each panel. Sections were incubated with non-specific IgG as a negative control (C) and there was no staining. The brown color represents localization of Ki67 protein.
Figure 3.5. ERα, MUC1 immunoreactivity in the uteri of WT and Bsg KO mice on day 1 pregnancy (n=3). (A) Localization of ERα in WT and Bsg KO (B) mouse uterus. (C) Localization of MUC1 protein in WT and Bsg KO (D) mouse uterus. The rectangles located at the bottom of the images are the enlarged views of the highlighted areas in each panel. Sections were incubated with non-specific IgG as a negative control (E) and there was no staining. The brown color represents the localization of each protein.
Figure 3.6. Ki67 immunoreactivity in the uteri of ovariectomized WT and Bsg KO mice treated with 200ng estradiol (n=3). (A) Localization of Ki67 protein in WT mouse uterus. (B) Localization of Ki67 protein in Bsg KO mouse uterus. The rectangles located in the bottom of the images are the enlarged views of the highlighted areas in each panel. The brown color represents the localization of each protein. Scale bar, 200 µm.
Figure 3.7. BSG immunoreactivity in the uteri of ovariectomized WT (A, C) and C/EBPβ KO mice (B, D) treated with 250ng estradiol (n=3). Uteri were collected at 22 and 24 hours following estradiol administration. Localization of BSG protein in WT mouse uterus at 22 hours (A), and 24 hours (C) following estradiol administration. Localization of BSG protein in WT mouse uterus at 22 hours (B), and 24 hours (D) following estradiol administration. Sections were incubated with non-specific IgG as a negative control (E) and there was no staining. The rectangles located in the bottom of the images are the enlarged views of the highlighted areas in each panel. The brown color represents the localization of each protein. The arrowhead represents the BSG negative epithelium. Scale bar, 200 µm.
References


Chapter Four

Basigin is Necessary for Normal Decidualization of Human Uterine Stromal Cells

Abstract

Decidualization of human endometrial stromal cells (HESCs) into decidual cells is carefully controlled, both spatially and temporally, by a balance of stimulatory and inhibitory signals from the uterine endometrium and is a prerequisite for successful implantation. Decidualization is associated with induction of a number of genes including wingless-type MMTV integration site family member (WNT4) and forkhead transcription factor forkhead box O1A (FOXO1). Basigin (BSG), a member of the immunoglobulin superfamily, is a multifunctional glycoprotein that plays an important role during implantation. The aim of the present study was to determine 1) whether BSG is required for HESCs decidualization; and 2) to identify some of the BSG-mediated molecular pathways involved in decidualization of HESCs. HESCs were transfected with small interfering RNAs targeting BSG gene expression. Expression of the decidualization markers Insulin-like growth factor-binding protein 1 (IGFBP1) and Prolactin (PRL) was significantly inhibited in cells with down regulated BSG expression. Silencing of BSG in HESCs also impaired expression of several MMPs. Microarray analysis revealed that both WNT4 and FOXO1 and its downstream targets are under the regulation of BSG during decidualization in HESCs. These results have provided new insights into the molecular pathways that regulate decidualization of human uterine stromal cells. Understanding the role of BSG during decidualization may help to explain the defects in decidualization-associated reproductive disorders of women.
Introduction

Successful establishment of pregnancy in humans and rodents depends on reciprocal interactions between the implantation-competent blastocyst and a receptive uterus. A remarkable maternal response to the implanting embryo is decidualization which involves the proliferation and differentiation of fibroblast-like endometrial stromal cells into large decidual cells that eventually form the maternal component of the placenta (Bany and Schultz, 2001; Chen et al., 2012; Dey et al., 2004; Hayashi et al., 2009). Decidualization provides the required environment for the embryo to survive and develop until formation and differentiation of the placenta are completed at approximately five days after the onset of implantation (Finn and McLaren, 1967). Decidual cells also serve an immunoregulatory role during pregnancy and regulate trophoblast invasion. Additional functions of the decidual cells are to support maternal blood vessel formation in order to perfuse and nourish the developing embryo (Ramathal et al., 2010b).

The Wingless-type MMTV integration site family member (WNT) signaling transduction pathway plays an important role in a number of developmental processes, including body axis formation, development of the central nervous system, axial specification in limb development and mouse mammary gland development. WNTs are highly conserved secreted glycoproteins and more than 15 different ligands have been reported previously in vertebrates (Akiyama, 2000; Polakis, 2000). WNTs modulate developmental and other processes through interaction with another family of proteins called the Frizzled homolog (FZD) receptors, which are seven-transmembrane, G
protein-coupled receptors that possess an extracellular cysteine-rich domain for WNT binding (Ramathal et al., 2010a). Beta-catenin (CTNNB1) is a key mediator of canonical WNT signaling and is critical for implantation and decidualization (Herington et al., 2007; Mohamed et al., 2005). Recently, it has been reported that WNT4, one of the WNT ligands, acts through activation of the β-catenin pathway to control the expression of FOXO1 and its downstream genes in regulating decidualization (Li et al., 2012). FOXO1, a member of the FOXO sub-family of Forkhead/winged helix family of transcription factors, is one of the earliest induced transcription factors in human endometrial stromal cells (HESCs) in response to cAMP signaling and is required for decidualization. FOXO1 has also been shown to be involved in the regulation of extracellular matrix remodeling (Takano et al., 2007).

Basigin (BSG) is abundant in the reproductive organs and plays essential roles in reproduction (Chang et al., 2004; Igakura et al., 1998; Kuno et al., 1998). In mice, it has been shown that BSG is expressed in both embryo and maternal uterus and is essential for successful implantation. In addition, WNT/β-catenin signaling is crucial for implantation and decidualization (Herington et al., 2007; Li et al., 2012; Mohamed et al., 2005). BSG acts, at least part, through the WNT/β-catenin signaling pathway (Sidhu et al., 2010). Therefore, the goals of this study were twofold: 1) to determine if BSG is essential for decidualization of HESCs. 2) to investigate the pathway that operates downstream of BSG upon silencing of BSG expression in decidualizing HESCs. I demonstrate that BSG is required for decidualization of HESCs. Silencing of BSG expression impaired the expression of WNT4, FOXO1 and its target genes in HESCs.
Materials and Methods

Human decidual tissues
Human uterine tissues were obtained from patients with elective terminations of first-trimester pregnancies (n=3). Human samples were collected and immediately processed for immunohistochemical analysis. All procedures were under approved protocols at John Stroger Jr. Hospital of Cook County and the Institutional Review Board of the University of Illinois at Chicago.

Immunohistochemistry
Human uterine tissues were fixed in 10% neutral buffered formalin for 24 hours. Tissues were then processed and embedded into paraffin blocks. Tissue blocks were sectioned at 5 µm and mounted on poly-L-lysine-coated slides. Antigen retrieval was performed by boiling sections in 10 mM citrate buffer (pH 6.0) for 10 minutes and endogenous peroxidase activity was quenched with methanol containing 0.3% hydrogen peroxide for 15 minutes. Non-specific binding was blocked with 5% normal rabbit serum in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 20 minutes. Slides were then incubated with a 1:25 dilution of goat polyclonal antibody against the intracellular domain of recombinant human BSG (rhEMMPRIN) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA in PBS at 4°C overnight. Non-specific goat IgG (2 µg/ml) in 1% BSA in PBS was used as a negative control. Slides were washed 3 times for 5 minutes each in PBS then incubated with biotinylated rabbit anti-goat IgG (Vector Labs, Burlingame, CA) diluted 1:100 with 1%BSA in PBS for 60 minutes at room temperature. Indirect detection of BSG was performed by incubation of sections for 45
minutes with avidin-biotinylated peroxidase complex (ABC), reacted with 0.2 mg/ml Metal-3, 3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO) in Tris-HCl buffer pH 7.6, for 3 minutes and finally counterstained with hematoxylin.

**Human endometrial stromal cell culture and in vitro decidualization model**

Telomerase-immortalized human endometrial stromal cells (HESCs) (Krikun et al., 2004) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin–Streptomycin (Cellgro, Manassas, VA) in a 5% CO₂ humidified incubators at 37°C. HESCs were then be treated with a hormone mixture containing 0.5 mM 8-bromo-cAMP, 1 µM progesterone and 10 nM 17β-estradiol (Sigma, St. Louis, MO) for 0-8 days (Li et al., 2007).

**Small interfering RNA (siRNA) transfection**

SiRNAs corresponding to human BSG (sense: CGUAGAUUCCCAUCAUACAtt; antisense: UGUAUGAUGGGAAUCUACGgg) were purchased from Ambion (Life technologies, Grand Island, NY). Silencer select negative control #1 (Life technologies) was used as a negative control under the same conditions. The BSG siRNA or negative control #1 were transfected into HESCs following the protocol of siLentFect™ Lipid (Bio-Rad, Hercules, CA). Briefly, 3 µl of siLentFect Lipid transfection reagent were mixed with 5 nM of siRNA or negative control #1 to form complexes and were dispersed into 6-well cell culture plates at 37°C. After complete knocking down BSG in HESCs (72 hours after adding siRNA), HESCs were treated with media containing estrogen, progesterone
and cAMP to induce decidualization. BSG siRNA and negative control #1 were also added every four days (day 0, 4). Total RNA and protein were harvested at each time point (day 0, 2, 4, 6, 8) for either Quantitative RT-PC or immunoblotting.

Cell Proliferation

HESCs were seeded at a density of 2.5 x 10^4 cells/well in 6-well plates and were cultured in the culture medium containing with DMEM/F12, 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin for 24 hours. HESCs were then transfected with either BSG siRNA or negative control #1. After 48 hours, the siRNA and negative control #1 were removed and cells were cultured in the culture medium for another 24 hours. Cells were then harvested and the number of cells was counted.

For tritiated thymidine incorporation arrays, HESCs were transfected with either BSG siRNA or negative control #1 for 48 hours. HESCs were then labeled with [³H] Thymidine (2 µCi/ml) (Perkin Elmer, Waltham, MA) at 37°C for another 24 hours. Cells were then harvested and counted in a scintillation counter.

RNA isolation and quantitative reverse transcription-PCR (qPCR)

Total RNA was extracted from HESCs using TRIzol™ (Life Technologies, Carlsbad, CA) according to the manufacture’s instructions. Two micrograms of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacture’s instructions. qPCR analyses were performed using TaqMan® Universal PCR Master Mix No AmpErase® UNG (Life Technologies).
The following 20x Assays-on-Demand™ Gene Expression Assay primer-probe sets from Applied Biosystems were used for my studies: Prolactin (*PRL*) (Hs00168730_m1), Insulin-like growth factor-binding protein 1 (*IGFBP1*) (Hs00236877_m1), and *POP4* (HS00198357_m1). Briefly, 4.5 µl of a 1:10 diluted cDNA sample was mixed with 5.5 µl of master mix (5µl of TaqMan Universal PCR Mix and 0.5 µl of 20X Assay on Demand) for a total volume of 10 µl per well in a MicroAmp optical 384-well reaction plate. Three experimental replicates were performed for each sample. qPCR amplification and quantitation was performed using the ABI 7900 sequence detection system for 40 cycles (95°C for 15 sec, 60°C for 1min). The comparative CT method (ΔΔCt) was used for quantification of gene expression. Relative fold differences in gene expression for all tested genes were normalized to the *POP4* endogenous control.

**Microarray analysis**

Total RNA was purified on day 6 of the decidualization following the protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA) and then utilized for microarray analysis, using the Agilent Human arrays (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed in the University of Illinois at Urbana-Champaign Biotechnology Center. Briefly, Microarray data preprocessing and statistical analyses were done in R using the limma package. Median foreground and median background values from the 4 arrays were read into R and any spots that had been manually flagged (-100 values) were given a weight of zero. The background values were ignored because investigations showed that trying to use them to adjust for background fluorescence added more noise.
to the data; background was low and even for all arrays, so no background correction was done.

The M-values (log2 ratio of Cy5/Cy3 for each spot) for each array were first normalized within each array using the loess method\(^3\) and then normalized between arrays using the scale method. Agilent's Human Gene Expression 4x44K v2 Microarray interrogates 27,958 genes using 33,128 probes spotted one time (1X) and 999 probes spotted ten times (10X) each. After normalizations and removal of the positive and negative control probes, the data were split in two and separate statistical models were fit for the 1X probes and the 10X probes. The models were nearly identical linear models from the limma package, both assessed the difference between SI and NC and controlled for overall dye effect, but the model for the 10X probes additionally adjusted for the replicate spots per probe. The results for all 34,127 probes were combined back together and the raw p-values were adjusted using the False Discovery Rate method separately for each comparison.

**Immunoblotting analysis**

15 ug of the cell lysates from each time point were loaded onto SDS-PAGE gels and transferred to Immobilon-P membrane (PVDF) (Millipore, Billerica, MA). Membranes were blocked in 5% nonfat dry milk and then probed with mouse anti human BSG (BD Pharmingen, San Jose, CA) at 1:2,000 dilution, mouse anti human MMP-2 (Calbiochem, Billerica, MA) at 1:1000 dilution and mouse anti human MMP-3 (R&D, Minneapolis, MN) at 1:100 dilution overnight at 4°C. The membranes were washed and
incubated with HRP-conjugated horse anti-mouse IgG antibody (Cell Signaling, Boston, MA) at 1:10,000 dilution for 60 min at room temperature. The bound secondary antibody was detected using SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburgh, PA). The same membranes were stripped and reprobed with anti-GAPDH antibody (Cell Signaling, Boston, MA) as a loading control.

**Densitometric analysis of immunoblotting**

Films developed after exposure to chemiluminescence were scanned, and images were analyzed using the ImageJ software from NIH (available at [http://rsbweb.nih.gov/ij/download.html](http://rsbweb.nih.gov/ij/download.html)). Quantitative measurements of BSG bands were normalized for loading differences using measurements of GAPDH. Graphs represent means and SEM.

**Statistical analysis**

The samples for this study were prepared from at least three independent cell cultures subjected to the same experimental treatments. Results are presented as the mean ± SEM. of at least three replicates. Statistical analyses were performed by ANOVA. Statistical significance was set at $P<0.05$.

**Results**

**BSG protein expression in human decidua**

Immunohistochemistry was carried out in order to determine if BSG is localized in human decidual cells. BSG was highly expressed in human decidual cells at day 60 of
pregnancy (Fig. 4.1A). No BSG immunoreactivity was observed in IgG controls (Fig. 4.1B).

**Silencing of BSG expression reduces stromal cell proliferation.**

Proliferation of stromal cells is the first step of decidualization. To examine the effect of BSG on human stromal cell proliferation, I employed RNA interference-mediated knockdown of its gene expression. HESCs were cultured and transfected with siRNA targeted specifically to BSG mRNA. In control experiments, cells were transfected with a scrambled siRNA that had no match with any mRNA of the human database. I observed that cells transfected with BSG siRNA exhibited an approximately 40% reduction in cell number compared with controls after 72 hours of treatment for BSG siRNA (Fig. 4.2A). Tritiated thymidine incorporation assays showed that the cells transfected with BSG siRNA exhibited around a 50% reduction in proliferation rate compared with controls after 72 hours of treatment for BSG siRNA (Fig. 4.2B).

**BSG is essential for decidualization of HESCs.**

To further determine the role of BSG in stromal cell decidualization, I, again, employed RNA interference-mediated knockdown of BSG gene expression. I observed that BSG protein levels in the cells transfected with BSG siRNA were knocked down to extremely low levels with little expression compared to controls (Fig. 4.3A, B). IGFBP1 and PRL are well-known markers of decidualization in human uterine stromal cells (Eyal et al., 2007; Li et al., 2007). My results showed that cells transfected with BSG siRNA showed a marked reduction in IGFBP1 and PRL mRNA expression compared with scrambled
siRNA treated controls (Fig. 4.3C, D). These results indicate that expression of BSG by human stromal cells is required in order to undergo normal decidualization.

**Silencing of BSG expression in decidualizing HESCs down regulates MMP expression.**

BSG is recognized as a potent inducer of MMPs (Gabison et al., 2005; Kataoka et al., 1993). MMP-2 and MMP-3 have been shown to be expressed in human uterine stromal cells (Braundmeier et al., 2006; Braundmeier and Nowak, 2006; Itoh et al., 2012). I investigated whether MMP-2 and MMP-3 levels were altered in HESCs in response to knock down of BSG. My results showed that both MMP-2 and MMP-3 protein levels were down regulated in BSG siRNA transfected HESCs compared with controls (Fig. 4.4). In addition, my microarray analysis showed that MMP-2, MMP-11, MMP-15 and MMP-19 mRNAs were all significantly down regulated in cells where I knocked-down BSG expression (Table 4.1).

**WNT4 expression is induced downstream of BSG signaling during decidualization**

WNT4 is essential in controlling uterine stromal cell decidualization (Li et al., 2007) and BSG has been reported to act through the WNT/ β-catenin signaling pathway (Sidhu et al., 2010). My microarray analyses revealed that the expression of WNT4 was markedly decreased in BSG siRNA transfected HESCs. Moreover, the expression levels of WNT2, WNT3, WNT5A, WNT5B and WNT7A were also down regulated in these cells (Table 4.2). I also observed that the expression of several Frizzled genes, FZD1, FZD4,
FZD9, and FZD10, which serve as WNT receptors were also significantly decreased in response to silencing of BSG expression (Table 4.2).

**FOXO1 is a downstream effector of BSG during decidualization of HESCs**

Previous studies have demonstrated a crucial role of FOXO1 in HESC decidualization. My microarray revealed that expression of the transcription factor FOXO1 was markedly down regulated in BSG siRNA transfected HESCs. Moreover, several of the previously reported downstream targets of FOXO1, such as Superoxide dismutase 2, mitochondrial (SOD2), BCL2-like 11 (BCL2L11), left-right determination factor 2 (LEFTY2), bone morphogenic protein and activin membrane-bound inhibitor (BAMBI), somatostatin (SST), decorin (DCN), and TIMP metallopeptidase inhibitor 3 (TIMP3) also showed down regulation in response to silencing of BSG expression (Table 4.3).

**Discussion**

Implantation triggers the proliferation followed by differentiation of endometrial stromal cells into large decidual cells. These decidual cells later either undergo apoptosis or form the maternal component of the placenta (Abrahamsohn and Zorn, 1993; Welsh and Enders, 1993). This process, called the decidual cell reaction or decidualization, provides the required environment for the embryo to survive and develop until complete placental differentiation is completed (Finn and McLaren, 1967). The purpose of the present study was to clarify the role of BSG in this complex process in human uterine stromal cells. Previous studies have shown that BSG is abundant in proliferative and undifferentiated cells (Biswas and Nugent, 1987; Chen et al., 2010; Chen et al., 2001;
Polette et al., 1997; Sameshima et al., 2000; Xiao et al., 2002). In mice, BSG is only expressed in undifferentiated deep stromal cells and predecidual cells, rather than decidual cells during embryo implantation (Chen et al., 2009). However, in this study I observed that BSG was highly expressed in human decidual cells during pregnancy. This distinct expression pattern led us to speculate that BSG protein might play a role in decidualization.

To test this hypothesis, I employed RNA interference-mediated knockdown of BSG gene expression to determine the effect of loss of BSG expression on proliferation and decidualization in vitro. I observed that HESCs transfected with BSG siRNA showed a significant reduction in proliferation. Moreover, cells transfected with BSG siRNA did not show the normal response to the decidualization regimen of estradiol, progesterone and cAMP displaying markedly reduced levels of IGFBP1 and PRL mRNA expression compared with scrambled siRNA treated controls. In addition, the results of the microarray analysis showed that expression of several important decidualization-associated genes such as WNT4 and FOXO1 were significantly decreased in HESCs treated with BSG siRNA. These results indicate that expression of BSG is required by human stromal cells to undergo normal decidualization.

FOXO1, a member of the FOXO sub-family of Forkhead/ winged helix family of transcription factors, is one of the earliest induced transcription factors in HESCs in response to cAMP/progesterone receptor (PGR) signaling. FOXO1 is critical in the induction of human decidualization. Silencing of FOXO1 significantly inhibits the
expression of IGFBP1 (Buzzio et al., 2006; Grinius et al., 2006; Takano et al., 2007). Recently, it has been reported that WNT4, one of the WNT ligands, acts through activation of the β-catenin pathway to control the expression of FOXO1 and its downstream genes thereby regulating decidualization (Li et al., 2012). Overexpression of WNT4 in HESCs leads to the elevation of both FOXO1 mRNA and protein levels. Moreover, several of the previously reported downstream targets of FOXO1 also showed enhanced expression in response to WNT4 stimulation. Surprisingly, a previous report indicated that down regulation of FOXO1 expression in HESCs leads to a suppression of WNT4 mRNA expression (Takano et al., 2007). This raises the interesting possibility that FOXO1, once induced downstream of WNT4, sustains the decidual phenotype of HESCs by maintaining the persistent expression of WNT4 via a positive feedback loop.

WNT signals are pleiotropic, with effects that include mitogenic stimulation, cell fate specification, and differentiation. WNT/β-catenin signaling is crucial for implantation and decidualization (Herington et al., 2007; Li et al., 2012; Mohamed et al., 2005). Previous studies have been shown that BSG regulates the canonical WNT/β-catenin signaling pathway in lung tumorigenesis. Increasing BSG levels in lung tumor epithelial cells leads to an enhancement in the metastatic potential of these cells. Moreover, inhibition of BSG drastically blocked the Wnt/β-catenin signaling pathway and inhibited several important parameters necessary for tumor progression, such as cell proliferation, cell migration and anchorage-independent growth (Sidhu et al., 2010).
In this study, I found that most of the WNT ligands and receptors were markedly decreased in BSG siRNA transfected HESCs. WNT4, a decidualization associated gene, showed a 95% reduction in response to silencing of BSG expression. Moreover, my microarray revealed that the expression of the transcription factor FOXO1 was also down regulated in BSG siRNA transfected HESCs. Several of the previously reported downstream targets of FOXO1, such as SOD2, BCL2L11, LEFTY2, BAMBI, SST, DCN, and TIMP3 also showed down regulation in response to silencing of BSG. Taken together, my findings support a role for BSG in the regulation of decidualization of human uterine stromal cells through the WNT4/ β-catenin-FOXO1 signaling pathway.

In summary, my studies have revealed an important link between BSG and the WNT/β-catenin signaling pathway in the peri-implantation uterus. I have found that BSG may act as a regulator of the WNT/β-catenin signaling pathway in human uterine stromal cells during decidualization. Inhibition of BSG using BSG siRNA markedly inhibited proliferation as well as expression of several important decidualization associated genes. Furthermore, signaling by BSG induces expression of WNT4, which acts by activating the β-catenin pathway to control the expression of FOXO1 and its downstream genes during decidualization (Fig. 4.5). My future studies will focus on detailing the molecular mechanisms by which the BSG-WNT4 pathway controls the process of decidualization.
Figure 4.1. Immunohistochemical analysis of BSG protein localization during pregnancy. (A) Decidua at day 60 of pregnancy (arrow indicates rounded decidual cells) show strong expression of BSG in the human decidual cells. (B) There was no positive staining observed in the IgG controls. The brown color represents localization of BSG.
Figure 4.2. Silencing of *BSG* expression inhibits stromal cell proliferation. HESCs were transfected with either *BSG* siRNA (5 nM) or scrambled siRNA (control) for 72 hours. Changes in cell number and incorporation of $[^3H]$-thymidine were determined. (A) Cell numbers of *BSG* siRNA treated cells were significantly decreased in comparison to the control cells. (B) DNA synthesis (thymidine incorporation) in *BSG* siRNA treated cells was significantly decreased in comparison to the control cells. NC: negative control (scrambled siRNA). Bars represent the fold change ± SEM of three independent experiments and the asterisk indicates statistical differences (P< 0.05).
Figure 4.3. Silencing of BSG expression inhibits decidualization. HESCs were transfected with either BSG siRNA (5 nM) or scrambled siRNA (control) and subjected to in vitro decidualization as described in Materials and Methods. (A) Immunoblotting analysis of BSG protein from HESCs transfected with BSG siRNA and scrambled siRNA. GAPDH served as the loading control. (B) Bar graph summarizing the densitometric analysis of the immunoblotting. (C) HESCs were harvested at indicated times after addition of the hormone regimen. Total RNA was isolated and subjected to qPCR using gene-specific primers for the decidualization markers IGFBP1(C) and PRL (D). Y-axis indicates fold expression. NC: negative control (scrambled siRNA). Bars represent the fold change ± SEM of three independent experiments and the asterisks indicate the statistical differences (P< 0.05).
Figure 4.4. Silencing of BSG expression reduces MMP production. HESCs were transfected with either BSG siRNA (5 nM) or scrambled siRNA (control) and subjected to *in vitro* decidualization as described in Materials and Methods. (A) Immunoblotting analysis of MMP-2 and MMP-3 proteins from HESCs transfected with BSG siRNA or scrambled siRNA. GAPDH served as the loading control. (B) Bar graph summarizing the densitometric analysis of MMP-2 immunoblotting. (C) Bar graph summarizing the densitometric analysis of MMP-3 immunoblotting. NC: negative control (scrambled siRNA). Bars represent the fold change ± SEM of three independent experiments and the asterisks indicate statistical differences (P< 0.05).
Figure. 4.5. BSG-WNT4-FOXO1 pathway in HESC decidualization. BSG regulates the expression of both WNT4 and FOXO1. WNT4 then functions via the β-catenin dependent signaling pathway to induce FOXO1 and its downstream genes to regulate HESC decidualization. FOXO1 may also be an essential maintenance factor for WNT4 expression.
Table 4.1. Silencing of BSG expression in decidualizing HESCs leads to down regulation of MMP expression.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene bank no.</th>
<th>Fold change</th>
<th>P value</th>
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<td>NM_004530</td>
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Table 4.2. Silencing of BSG expression in decidualizing HESCs leads to down regulation of WNT and FZD family members expression.

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<th>Gene Name</th>
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<td>WNT5B</td>
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Table 4.3. Silencing of *BSG* expression in decidualizing HESCs leads to down regulation of expression of *FOXO1* and its target genes.

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References


84


Chapter Five

Sterility in Basigin Null Mutant Male Mice May Be Due to Impaired Interactions Between Gametes and Sertoli Cells

Abstract

Basigin (BSG) is a multifunctional glycoprotein that plays an important role in male reproduction since male knockout (KO) mice are sterile. The Bsg KO testis lacks elongated spermatids and mature spermatozoa, a phenotype similar to that of alpha-mannosidase IIx (MX) KO mice. MX regulates formation of N-acetylglucosamine (GlcNAc) terminated N-glycans that participate in germ cell-Sertoli cell adhesion. Results showed that Bsg KO spermatocytes displayed normal homologous chromosome synapsis and progression to the midpachytene stage. Both meiosis I (MI) and meiosis II (MII) spermatocytes were detected in the KO preparations. However, expression of the acrosome marker SP-10 was extremely low in germ cells of Bsg KO mice indicating that spermatogenesis in Bsg KO mice was arrested at the round spermatid stages. I observed a large increase in the number of germ cells undergoing apoptosis in Bsg KO testes. Using lectin blotting, I determined that GlcNAc terminated N-glycans are linked to BSG. GlcNAc terminated N-glycans were significantly reduced in Bsg KO testes. These observations indicate that BSG may act as a germ cell-Sertoli cell attachment molecule. Loss of BSG significantly reduced adhesion between GC-2 and SF7 cells. Moreover, wild type testes showed strong expression of N-cadherin (CDH2) while expression was greatly reduced in the testes of Bsg KO mice. In addition, the integrity of the blood-testis barrier (BTB) was compromised and flow of fluid from the testis was reduced in Bsg KO testes. In conclusion, although Bsg KO spermatogonia
can undergo normal progression to the spermatocyte stage, BSG-mediated germ cell-Sertoli cell adhesion via GlcNAc terminated N-glycans appears to be necessary for integrity of the BTB and spermatocyte progression to mature spermatozoa.

Introduction
Spermatogenesis is the process of producing sperm (haploid) from initially undifferentiated spermatogonia (diploid). During spermatogenesis, there are a group of somatic cells named Sertoli cells that have direct contact with all the different types of germ cells in the seminiferous tubules. Sertoli cells play a major role in the regulation of germ cell differentiation and migration. First, these cells form the blood-testis barrier (BTB) in the seminiferous epithelium near the basement membrane which protects the postmeiotic germ cells from the host immune system in order to avoid attack from the production of anti-sperm antibodies against germ cell specific antigens (Dym and Fawcett, 1970; Morrow et al., 2009; Wong et al., 2010). The BTB has been shown to be composed of tight junctions (TJ), basal ectoplasmic specializations (basal ES, a testis-specific type of adherens junction), gap junctions (GJ) and desmosome-like junctions. Several adhesion molecules have been identified to play an important role in the functioning of these junctions including claudins and occludins (TJ); coxsacckie and adenovirus receptor (CXADR), N-cadherin (CDH2) and β-catenin (basal ES); connexin43 (CX43) (GJ); desmoglein-2 and desmocollin-2 (desmosome-like junction) (Cheng and Mruk, 2009; Komljenovic et al., 2009; Pointis and Segretain, 2005; Wong et al., 2008). Knockouts generated against specific junction proteins such as claudin 11 (CLDN11) (Gow et al., 1999) can cause sterility in the male mouse, which emphasizes
the role these junction proteins play in the appropriate functioning of the BTB and also prove that an intact BTB is necessary for successful male fertility.

Sertoli cells are also responsible for the formation and secretion of seminiferous tubule fluid (STF) which consists of a number of products that are involved in spermatogenesis such as transport and binding proteins, proteases and protease inhibitors, hormones and growth factors (Russell and Griswold, 1993; Skinner and Griswold, 2005). STF is important for maintaining the nutritional and hormonal microenvironment necessary for the viability and differentiation of germ cells during spermatogenesis. It also provides the milieu for developing spermatozoa and the vehicle to transport them from the testis (Rato et al., 2010; Richburg et al., 1994). Furthermore, there is strong evidence that some ligands secreted by Sertoli cells can modulate gene expression in germ cells during spermatogenesis (Tsuruta and O'Brien, 1995). Sertoli cells also communicate with germ cells through direct cell-cell contact and paracrine interactions. The loss of these interactions between Sertoli cells and germ cells leads to the failure of spermatogenesis. For example, oligosaccharide 310.11 which is a N-acetylglucosamine (GlcNAc) terminated tri-antennary and fucosylated N-glycan structure has been shown to play a key role in germ cell adhesion to Sertoli cells. A 50% reduction in expression of this N-glycan structure results in almost complete suppression of fertility (Akama et al., 2002). Although this specific oligosaccharide plays a key role in spermatogenesis, the glycoprotein that the oligosaccharide is attached to is still unknown.
Basigin (basic immunoglobulin superfamily; BSG) is a transmembrane glycoprotein rich in N-glycans that belongs to the immunoglobulin superfamily (Miyauchi et al., 1990; Tang et al., 2004). This molecule has been identified independently by different laboratories and is also known as EMMPRIN, CD147 and M6 in human, GP42 in mice, HT7 and neurothelin in chicken and OX-47, MC31 and CE9 in the rat (Nehme et al., 1995; Sameshima et al., 2000; Schlosshauer and Herzog, 1990; Seulberger et al., 1992; Wakayama et al., 2000). The molecular weight of BSG lies in a range between 43 kD and 66 kD due to variable glycosylation whereas the molecular weight of the core protein is around 27 kD (Fossum et al., 1991; Miyauchi et al., 1990; Seulberger et al., 1992). BSG is highly expressed in reproductive tissues and plays a crucial role in both male and female reproduction (Chen et al., 2010; Igakura et al., 1998; Kuno et al., 1998). In the mouse testis, BSG is expressed in Sertoli cells, Leydig cells and strongly expressed in spermatocytes and spermatids (Chen et al., 2010; Igakura et al., 1998).

Bsg gene knockout (KO) males, created by targeted disruption, are sterile due to the failure of spermatogenesis (Igakura et al., 1998). However, the underlying mechanisms of Bsg action in mammalian spermatogenesis still remain unclear. The goals of the current study of Bsg gene null mutant (-/-) testes were: 1) to determine the effect of loss of BSG on the transition of spermatocytes to spermatids; 2) to investigate the importance of BSG in maintenance of the BTB; and 3) to determine whether BSG is the predominant protein that binds N-glycans in the testis.
Materials and Methods

Animals

Animals used in this research were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Illinois. The wild-type C57BL6/J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were housed under temperature and light controlled conditions (12 h light: 12 h dark) with free access to food and water. C57BL6/J heterozygous (Bsg +/-) mice were a kind gift from Dr. Takashi Muramatsu, Department of Biochemistry, Nagoya University School of Medicine, Japan. Heterozygote breeding was carried out in the animal facility of the University of Illinois. At 3 weeks of age, male offspring were anesthetized by isoflurane inhalation (Attane; Minrad, Bethlehem, PA) and tail snips were collected for genotyping. DNA was extracted from the snips using REDExtract-N-Amp (Sigma), and genotypes determined by PCR using primers for Bsg and neomycin (Igakura et al., 1998). Offspring with the Bsg -/- phenotype were maintained under the same conditions as those for the WT mice.

Tissue collection

The wild type (WT) and Bsg KO mice (7 days, 12–24 weeks or 15-17 months of age) were euthanized with carbon dioxide and testes collected immediately. Tissues for histology were fixed in modified Davidson’s solution (Electron Microscopy Sciences, Hatfield, PA) or 4% paraformaldehyde (Electron Microscopy Sciences) in PBS overnight and processed for paraffin embedding. Tissues for Western and lectin blotting analyses were snap-frozen in liquid nitrogen and stored at -80°C until use.
Radioimmunoassay

Blood samples were collected from WT and Bsg KO mice (n=3) and serum samples were stored at -80°C until the assays were performed. Testosterone levels were measured using a radioimmunoassay performed by the University of Virginia Core Ligand and Assay Laboratory. The limit of detection for this assay was 0.1 ng.

Cell culture

GC-2 cells (Hofmann et al., 1995) are an immortalized spermatocyte cell line and were purchased from the American Type Culture Collection (CRL-2196) (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (ATCC) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1% L-glutamine (Cellgro, Manassas, VA) and 1% Penicillin–Streptomycin (Cellgro, Manassas, VA) in a 5% CO₂ humidified incubators at 37°C.

SF7 cells (Hofmann et al., 1992) are an immortalized Sertoli cell line and were a gift from Dr. Hofmann at University of Illinois. Cells were cultured in DMEM/F12 (Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin in a 5% CO₂ humidified incubators at 37°C.

Germ cell isolation and spermatocyte enrichment

Testes were removed from both WT and Bsg KO mice, detunicated and digested in 0.5 mg/ml collagenase (Sigma, St. Louis, MO) in Krebs-Ringer bicarbonate solution (KRB) [120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM
MgSO$_4$·7H$_2$O, 1.3 mM CaCl$_2$, 11 mM glucose, 1 X essential amino acids, 1 X nonessential amino acids] at 32°C for 20 min, followed by digestion in 0.5 mg/ml trypsin (Sigma) containing 20 mg/ml DNase I in KRB at 32°C for 13 min. After digestion the cell suspension was filtered through an 80-µm mesh filter and washed three times in KRB. Germ cells were then processed as described below.

**Cytological analyses**

For immunofluorescence analysis, cells were collected by centrifugation, surface-spread in wells of multispot microscope slides (Shandon, Pittsburgh, PA) and fixed following the procedure previously described (Cobb et al., 1999b; Cobb et al., 1997). Prior to antibody labeling, slides were washed three times in washing/blocking buffer (0.3% BSA, 1% goat serum in phosphate-buffered saline, pH 7.4); the second wash included 0.05% Triton-X 100. After draining, the slides were incubated with primary antibodies. Antibodies and dilution used were: rabbit anti-SYCP1 and SYCP3 (Novus, Littleton, CO) used at 1:100 dilution; guinea pig anti-H1T (Cobb et al., 1999a) at 1:500; rabbit anti-$\gamma$H2AX (Millipore, Billerica, MA) at 1:200; Secondary antibodies against rabbit, rat or mouse IgG and conjugated with Alexa 594 or 488 (Molecular Probes) were used at 1:500 dilution. Images were acquired with a Leica DMRXE epifluorescence microscope equipped with a 100X plan-neofluar oil-immersion objective lens and a triple filter (set no. 61000V2 BS&M, Chroma Technology, Rockingham, VT) for simultaneous visualization of green (Alexa 488), red (Alexa 594), yellow (Alexa 488 + Alexa 594) and blue (DAPI) fluorescence. The microscope was linked to a Micromax cooled CCD camera (RS Princeton Instrument) with a high-speed shutter driven by a Sutter Lambda
10-2 (Sutter Instrument) and Metamorph software (Universal Imaging Corporation) to capture the images.

**Histology, immunohistochemistry and immunofluorescence**

Paraffin embedded tissues were sectioned at 4 µm and mounted on poly-L-lysine-coated slides. Sections were stained using the periodic acid-Schiff (PAS) reaction (Cerri and Sasso-Cerri, 2003) and hematoxylin for histological observation. After deparaffinization and rehydration, sections for immunostaining were boiled in 10 mM citrate buffer (pH 6.0) for 30 minutes to promote antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide for 15 minutes. After blocking with 2% normal serum (Vector Laboratories, Burlingame, CA) in PBS/Tween 20 (PBS/T) for 60 minutes, sections were incubated at 4°C overnight with: 2 µg/ml anti-BSG (R&D, Minneapolis, MN), anti-TRA 98, 1:1000 (a kind gift of Hiromitsu Tanaka, Osaka University, Japan), anti-SP-10, 1:400 (a kind gift of Dr. P. Reddi, University of Virginia), 0.2 µg/ml anti- CXADR (Santa Cruz, Santa Cruz, CA), 1 µg/ml anti-CX43 (Cell Signaling Technology, Danvers, MA), 2 µg/ml anti-CDH2 (Santa Cruz), and 0.33 µg/ml anti- CLDN11 (abcam, San Francisco, CA). Isotype control IgG was applied as a negative control. After washing in PBS/T, sections were incubated with secondary antibody either conjugated with CY3 at 1:1000 with PBS/T (Jackson ImmunoResearch, West Grove, PA) or biotin (Vector Laboratories) diluted at 1:200 with PBS/T for 60 minutes at room temperature. Sections for immunohistochemistry were then incubated in ABC solution (Vector Laboratories) for 30 minutes, reacted with metal-3,3'-diaminobenzidine (DAB) (Sigma) for minutes, and counterstained with hematoxylin.
Sections for immunofluorescence were then mounted using Vectashield (Vector Laboratories).

**Tunel staining**

Detection of DNA fragmentation of apoptotic cells was performed by using an ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore). Briefly, after deparaffinization and rehydration, sections were permeabilized by incubation with proteinase K (20 mg/ml) for 15 minutes. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide in PBS for 5 minutes. After a 10 second incubation in equilibration buffer, sections were incubated in the terminal deoxynucleotidyl transferase (TdT) labeling reaction mix for 1hr at 37°C. After a 10 minute wash in stop/wash buffer, sections were incubated with anti-digoxigenin and finally developed in DAB. For the negative controls, sections were processed without TdT enzyme in the labelling reaction mix. Sections were counterstained with 0.5% (w/v) methyl green for 10 min at room temperature.

**Barrier function assays**

The permeability of the BTB was assessed using the method developed by Chen et al. (Chen et al., 1997). Briefly, WT and BsgKO mice (12 weeks old) were anesthetized using ketamine/xylazine (2mg/0.2mg). Testes were exposed by a small incision through the scrotal layer. Next, a small opening in the tunica albuginea was gently created with fine forceps and 20ul of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Fisher Scientific, Pittsburgh, PA) were injected using a Hamilton syringe into the interstitium of one testis. The other testis was injected with 20ul of PBS as a control. After 30 min the animals
were euthanized by cervical dislocation, and the testes were immediately removed and fixed in 4% paraformaldehyde. Testes were then processed for paraffin embedding. The slides were deparaffinized, rehydrated, boiled in 10 mM citrate buffer (pH 6.0) for 30 minutes and incubated with Fluorescein Isothiocyanate (FITC) conjugated streptavidin (Life Technologies) for 60 minutes. Slides were then mounted with vectashield (Vector Laboratories).

**Lectin histochemistry**

Tissue sections were deparaffinized in xylene three times for a total of 60 minutes. The sections were rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide for 20 minutes. After blocking with 1% BSA in PBS/T for 60 minutes, sections were incubated at 4ºC overnight with 10µg/ml biotinylated *Griffonia simplicifolia* agglutinin (GSA) II (Vector Laboratories). GSA II that was preincubated with N-acetylglucosamine (Sigma) was applied as a negative control. After washing in PBS/T, sections were incubated in ABC solution (Vector Laboratories) for 30 minutes, reacted with DAB for 3 minutes, and counterstained with hematoxylin.

**Immunoprecipitation**

Tissues were homogenized and proteins extracted using TNE buffer (1% NP 40, 10mM Tris pH 8, 1mM EDTA). 500 µg of protein were incubated with 2 µg of goat polyclonal antibody against mouse BSG (R&D) or goat IgG for 60 minutes at 4ºC. Protein G plus-Agarose (Santa Cruz) was added into each protein sample overnight at 4ºC.
Immunoprecipitates were collected by centrifugation at 2500 rpm at 4°C and washed with RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS and proteinase inhibitor). The protein was removed from the beads by boiling for 5 minutes in 1X LSB (2% SDS, 10% glycerol, 6.25mM Tris pH 6.8). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.

**Immunoblotting analysis**

Membranes were blocked in 5% nonfat dry milk and then probed with 0.2 µg/ml goat polyclonal antibody against mouse BSG (R&D) overnight at 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated donkey anti-goat IgG antibody (abcam) at 1:5000 dilution for 60 minutes at room temperature. The bound secondary antibody was detected using SuperSignal West Pico Chemiluminescent Substrate kit (Fisher Scientific).

**Lectin blotting analysis**

Membranes probed for immunoblotting analysis were stripped with Restore Western Blot Stripping Buffer (Fisher Scientific). After blocking in 1X Carbo-free blocking buffer (Vector Laboratories) for an hour, the membranes were incubated with 1µg/ml GSA II overnight at 4°C. Membranes were then incubated in 0.2µg/ml Immunopure Avidin Hoseradish Peroxidase Conjugated (avidin-HRP) (Fisher Scientific) for 1 hr. The bound avidin-HRP was detected using SuperSignal West Pico Chemiluminescent Substrate kit (Fisher Scientific).
Small interfering RNA (siRNA) transfection and in vitro assay for germ cell-Sertoli cell adhesion

Methods for mouse spermatogenic cells (GC-2) and Sertoli cell (SF7) adhesion assays (Akama et al., 2002) were performed as previously described with the following modifications. SiRNAs corresponding to mouse Bsg (sense: GGCUGUACAUUGAGCAGAAtt; antisense: UUCUGCUCAAUGUACAGCCtc) were purchased from ambion (Life technologies, Grand Island, NY). The siRNA was transfected into the GC-2 cells following the protocol of siLentFect™ Lipid (Bio-Rad, Hercules, CA). Briefly, 3 µl of siLentFect Lipid transfection reagent was mixed with 10 nM of siRNA to form complexes and dispersed into 6-well cell culture plates at 37°C for 48 hours. Silencer select negative control #1 (Life technologies) was used as a negative control under the same conditions.

The GC-2 cells were then labeled with [³H] Thymidine (2 µCi/ml) (Perkin Elmer, Waltham, MA) at 37°C for another 24 hours. 4.2X10⁵ [³H]-labeled GC-2 cells were added to the SF7 cells and incubated at 37°C for 4 hours. After washing unbound cells with PBS, radioactivity remaining on the SF7 cells was counted in a scintillation counter.

Statistical analysis

The samples for this study were prepared from at least replicates subjected to the same experimental treatments. Results are represented as mean ± SEM. Differences between groups were examined using Student’s t-test. Statistical significance was set as $P<0.05$. 

97
Results

Spermatogenic arrest in Bsg-null mouse testes

The testes of Bsg KO mice were smaller than those of WT mice (Fig. 5.1A) and the testis to body weight ratio of the KO mice was significantly less than that of WT mice (Fig. 5.1B). Testosterone levels were determined for WT and Bsg KO male mice. The values ranged widely from 0.2-1.2 ng/ml for both groups and were not different from one another. I next examined the histology of both Bsg KO and WT testes. Both pachytene spermatocytes (Fig. 5.1C, arrowhead) and round spermatids (Fig. 5.1C, arrow) lost their intercellular bridges and formed multinucleated giant cells in contrast to WT testes where none were found. Moreover, there were a lot of abnormal chromatin patterns, with micronucleus formation, in the pachytene spermatocytes as they enter meiotic division (Fig. 5.1D, arrowhead) and significant cell death in the tubules was observed (Fig. 5.1E, arrowhead). I confirmed previous studies showing a reduction in the number of spermatogenic cells in Bsg KO mice (Igakura et al., 1998), as null mutant testes exhibited a complete loss of elongated spermatids and mature spermatozoa. I used a TUNEL assay to evaluate apoptosis of germ cells in Bsg KO mice. As shown in Figure 5.1F, there was a marked increase in the number of apoptotic cells in the testes of Bsg KO mice compared to WT testes. The position of these apoptotic cells in the seminiferous epithelium as well as their morphology indicated that they were spermatocytes.

The diameter of seminiferous tubular lumen in Bsg KO testes was 65% smaller than that of WT testes (Fig. 5.1G). I also confirmed previous studies showing the diameter of
seminiferous tubule in Bsg KO testes was significantly smaller than that of WT testes (~30%) (Toyama et al., 1999).

Because previous studies had not determined the progression of meiosis or whether meiotic division occurred in Bsg-null testes, I used classical markers for meiotic progression in immunofluorescence analyses. Antibodies that recognized SYCP1 (the central element of the synaptonemal complex), SYCP3 (the lateral element of the synaptonemal complex), phosphorylated histone H2AFX (commonly known as gH2AX), a histone marker of DNA double-strand breaks, and H1T (a male germ-cell specific histone marker for spermatocytes that have reached the mid-pachytene point of meiotic prophase) were used to assess whether the spermatocytes of Bsg KO males entered and progressed through meiosis (Costa et al., 2005; Inselman et al., 2003). My results showed that male germ cells of Bsg KO mice displayed normal patterns of chromosome dynamics (Fig. 5.2), exhibiting homologous chromosome synapsis (Fig. 5.2D, E) as well as normal labeling of the X-Y bivalent (Fig. 5.2J), which, as in WT germ cells, is the only unpaired chromosome (Fig. 5.2C, F). As seen in Figure 5.2K, Bsg KO spermatocytes reached the mid-pachytene stage, a time at which competence to enter meiotic division arises. This was confirmed by the presence of MI spermatocytes (Fig. 5.2L). After chromosomes underwent desynapsis at the end of meiotic prophase and entered the MI division phase, SYCP1 labeling disappeared, and SYCP3 labeling was observed at the centromeric ends of the chromosomes (Fig. 5.2I, L). In addition, some meiosis II spermatocytes were also observed in the KO preparations (data not shown).
To determine whether post-meiotic germ cell are present in Bsg KO mice, I immunostained adult testis sections for the acrosome marker SP-10 (officially known as ACRV1) (Herr et al., 1990; Reddi et al., 1999). As shown in Figure 5.3A, WT spermatids had a large arc-shaped acrosome with a distinctive pattern of SP-10 expression, but SP-10 expression was extremely low in germ cells in the seminiferous tubules of Bsg KO mice (Fig. 5.3B). I next used immunoblotting to quantify the level of SP-10 in both WT and Bsg KO testes. My results showed that SP-10 was strongly expressed in WT testes. However, the level of SP-10 in Bsg KO testes was too low to detect which corresponded with my immunohistochemistry results (Fig. 5.3C and D).

**Abnormalities in aged mouse testes caused by loss of BSG expression**

Numerous vacuoles were observed within the seminiferous epithelium of Bsg KO testes of aged mice (Fig. 5.4C, arrowhead) in contrast to WT (Fig. 5.4A) and younger Bsg KO (Fig. 5.4B) testes. Some areas of the seminiferous epithelium showed a significant loss of spermatogonia and failure of spermatogenesis (Fig. 5.4D). In some tubules I also observed Sertoli cells clumped in the lumen, appearing to have lost their attachment to the basement membrane (Fig. 5.4E, arrowhead). The lumen of Bsg KO efferent ductules contained degenerating sloughed germ cells and areas of aggregated debris (Fig. 5.4G, arrowhead) in contrast to WT (Fig. 5.4F). The epididymal lumen was mostly devoid of germ cells but some luminal debris was present with occasional degenerating germ cell
BSG protein expression in the mouse testes

Immunohistochemistry and immunofluorescence analysis were carried out in order to determine the localization of BSG in the mouse testes. BSG was highly expressed in the mouse testes and strong BSG immunoreactivity was observed in spermatocytes and spermatids (Fig. 5.5A, B). Type A spermatogonia can typically be observed in mouse testes as early as day 7-8 of age (Bellve et al., 1977). I therefore analyzed tissue sections from day 7 mouse testes to determine whether BSG is expressed in spermatogonia. Tra 98, a germ cell marker (Tanaka et al., 1997), was used to identify germ cells. My results showed that spermatogonia expressed BSG (Figure 5.5C). No BSG immunoreactivity was observed in sections from Bsg KOs (Fig. 5.5D), confirming the specificity of the antibody for mouse BSG.

Integrity of the BTB and expression of the BTB related adhesion molecules CXADR, CX43, CLDN11 and CDH2 in the testes of WT and Bsg KO mice

I next investigated the integrity of the BTB in Bsg KO testes by injecting a biotin tracer into the testes. In the WT testes the biotin tracer was restricted to the interstitium and the basal compartment of the seminiferous tubules (Fig. 5.6A, B). In contrast, in Bsg KO mice, the biotin tracer in some tubules penetrated beyond the BTB and surrounded early pachytene spermatocytes, but did not enter the lumen (Fig. 5.6E, F, arrowhead).

I therefore carried out immunohistochemical analysis of both WT and Bsg KO testes to determine the expression pattern of some well-known BTB-related adhesion proteins. I observed that CXADR immunoreactivity was strongly expressed along the basal
compartment of the seminiferous tubules of WT mice (Fig. 5.6C, arrowhead). A very similar localization pattern of CXADR protein was also observed in Bsg KO testes (Fig. 5.6G). CX43 immunoreactivity in WT testes was observed in spermatocytes and round spermatids. Weak immunoreactivity was detected in elongated spermatids but was undetectable in spermatogonia. A very strong immunoreactivity was detected at the site of the BTB (Fig. 5.6D, arrowheads). The localization pattern of CX43 protein in Bsg KO testes was very similar (Fig. 5.6H). CLDN11 immunoreactivity was detected at the site of the BTB of the seminiferous epithelium in both WT and Bsg KO testes and formed an almost continuous ring of expression (Fig. 5.6I, L).

I also examined expression of CDH2 and saw a significant alteration in expression in Bsg KO testes. CDH2 immunoreactivity in WT testes was observed at the site of cell-cell contacts between Sertoli cells and spermatogenic cells (Fig. 5.6J). However, whereas WT testes showed strong expression of CDH2 along the basal compartment of the seminiferous tubule (Fig. 5.6J, arrowheads), CDH2 expression was greatly reduced in the basal compartment of the seminiferous tubules (the site of the BTB) of Bsg KO mice (Fig. 5.6M). No signal was detected in the negative control sections (Fig. 5.6K, N).

**GlcNAc terminated N-glycans localization in testes of WT and Bsg KO mice**

GlcNAc terminated tri-antennary and fucosylated N-glycan structures have been shown to play a key role in germ cell-Sertoli cell adhesion (Akama et al., 2002). Therefore, I carried out lectin histochemistry analysis on both WT and Bsg KO testes using *Griffonia simplicifolia* agglutinin (GSA) II which recognizes GlcNAc terminated N-
glycans (Akama et al., 2002). I found that the expression pattern of GlcNAC terminated carbohydrates showed remarkable differences between WT and Bsg KO testes. In WT testes, spermatogenic cells were strongly stained with GSA II (Fig. 5.7A). In contrast, immunostaining in the Bsg KO testes was greatly decreased (Fig. 5.7B). No signal was found in the negative control sections (Fig. 5.7C).

Determining whether GlcNAC terminated N-glycans carbohydrates are linked to BSG.

BSG is a highly glycosylated protein rich in N-glycans (Miyauchi et al., 1990; Tang et al., 2004). Lectin blotting analysis was carried out to determine whether GlcNAC terminated N-glycans are one of the carbohydrates linked to BSG. I first immunoprecipitated protein lysates from mouse testes using a BSG antibody and then performed immunoblotting using this same BSG antibody to confirm that I had successfully pulled down BSG protein (Fig. 5.8). No BSG signal was observed from the IgG control, confirming the specificity of the antibody for mouse BSG. These membranes were then stripped and reprobed with GSAII. My lectin blotting clearly showed the presence of GlcNAC terminated carbohydrates corresponding with the BSG protein. These results confirmed that GlcNAC terminated N-glycans are one of the carbohydrates linked to BSG.

*Bsg* knockdown reduces adhesion of spermatocytes to Sertoli cells

In order to determine whether BSG plays a role in adhesion of spermatogenic cells to Sertoli cells, I performed an *in vitro* adhesion assays with GC-2 and SF7 cells transfected with *Bsg* siRNA. As shown in Figure 5.9A, the expression of BSG protein
was knocked down completely in GC-2 cells transfected with Bsg siRNA, but not in the negative control group 72 hours after transfection. Knockdown of Bsg expression of in GC-2 cells resulted in a significant reduction in adhesion between GC-2 and SF7 cells compared with controls (Fig. 5.9B).

**Discussion**

BSG is a transmembrane glycoprotein that is expressed in several reproductive tissues and plays an essential role in both male and female fertility (Igakura et al., 1998; Kuno et al., 1998). Previous studies have shown by microscopic analyses that most Bsg KO spermatocytes are arrested and degenerate at the metaphase of the first meiosis, with only a small number of germ cells differentiating into step 1 spermatids (Igakura et al., 1998; Toyama et al., 1999). Although some metaphase spermatocytes are present in Bsg KO testes, it was important to examine the key events of meiotic division more carefully in Bsg KO testes. My results showed that Bsg KO spermatocytes undergo normal homologous chromosome synapsis and progression to the metaphase of first meiosis as demonstrated by immunofluorescent labeling of chromosomes with SYCP1, SYCP3, H1T and γH2AX. However, I observed a number of abnormal chromatin patterns in the pachytene spermatocytes as they enter meiotic division, and significant cell death was observed in the tubules. It appears that these abnormalities occur without alterations in the expression of the primary genes regulating meiosis. Furthermore, to pinpoint the exact point of arrest in Bsg KO mice, I used an antibody to the acrosome marker SP-10 to identify spermatids in adult testes sections. Mouse spermiogenesis is divided into 16 steps according to the shape of the developing
acrosome. These stages are: Golgi phase, steps 1-4; cap phase, steps 5-7; acrosome phase, steps 8-12; and maturation phase, steps 13-16 (Russell, 1990; Segretain and Roussel, 1988). My results showed that spermatogenesis in Bsg KO mice is arrested right after the completion of meiosis, most likely at the early round spermatid stages (step 3 or 4), before any spermatid differentiation occurred. Moreover, I found that there was a large increase in the number of spermatocytes undergoing apoptosis in Bsg KO testes. These results are consistent with previous reports regarding apoptosis (Chen et al., 2012; Igakura et al., 1998; Toyama et al., 1999). Taken together, I conclude that BSG is crucial in the development/differentiation of round spermatids.

Spermatogenesis begins with mitosis of spermatogonia and formation of early spermatocytes. When I examined testes of old Bsg KO mice (around 15 month old), I observed that numerous vacuoles had formed within the seminiferous epithelium and pachytene spermatocytes showed abnormal nuclei and cell death. In some tubules, Sertoli cells were observed clumped in the lumen, appearing to have lost their attachment to the basement membrane. Moreover, some areas of the seminiferous epithelium showed significant loss of spermatogonia and failure of spermatogenesis. The phenotypes described above were not found in testes of aged WT or young Bsg KO males. Thus, in older KO males I observed a more severe phenotype suggesting that over time the impact of loss of BSG on spermatogenesis becomes more severe and cumulative.

The Sertoli cell is known to secrete fluid to form a seminiferous tubular lumen (Russell
et al., 1989). The lumen size in Bsg KO testes was 65% smaller than that of WT testes. Furthermore, the lumen of Bsg KO efferent ductules contained degenerating sloughed germ cells and areas of aggregated debris, suggestive of stagnation due to reduced flow of fluid from the testis. Taken together, these results support that BSG may also regulate the secretion of seminiferous tubule fluid from Sertoli cells during spermatogenesis.

Injection of biotin tracer is commonly used to determine the integrity of the BTB (Chen et al., 1997). Biotin tracer was restricted to testicular interstitium and the basal compartment of the seminiferous in WT testes whereas in Bsg KO testes, biotin tracer was localized within the interstitial space and up to the third layer of spermatocyte/round spermatids. This altered pattern of biotin localization indicates that the integrity of the BTB is compromised in Bsg KO testis. I further investigated this finding by analyzing immunolocalization of several BTB component proteins.

The junctional proteins CXADR, CX43, claudins, and CDH2 are important for maintenance of BTB integrity (Cheng and Mruk, 2009; Komljenovic et al., 2009; Pointis and Segretain, 2005; Wong et al., 2008). I did not observe any difference in localization of CXADR, CX43 or CLDN11 between WT and Bsg KO testes. However, the expression of CDH2 was greatly reduced at the basal compartment of the seminiferous tubule (the site of the BTB) in Bsg KO mice. CDH2 belongs to a family of calcium-dependent, homophilic cell adhesion molecules that are expressed in a number of tissues (Gumbiner, 2005). CDH2, along with β-catenin, is one of the component
proteins of the BTB (Cheng and Mruk, 2009; Elkin et al., 2010; Lee et al., 2003). Studies have shown that CDH2 is also involved in spermatogenesis. It mediates Sertoli cell-spermatogenic cell adhesion (Newton et al., 1993), and may also facilitate germ cell migration during spermatogenesis (Chung et al., 1998). Since the expression of CDH2 was greatly reduced in the testes of Bsg KO mice this suggests that BSG may regulate the expression of CDH2 either directly or indirectly. Taken together, my data show that BTB integrity is compromised but not destroyed completely in Bsg KO testes. This may be due to the presence of several other junction proteins such as CXADR, CX43 and CLDN11 that are also known to maintain BTB integrity and whose expression is not altered in Bsg KO testes. My results show, for the first time, that loss of BSG expression and reduced expression of the basal ES protein CDH2 disrupts Sertoli cell BTB integrity.

N-glycans are branched carbohydrates that are attached to the amide nitrogen of asparagine residues of peptides. There are three broad classes of N-glycans: high mannose, hybrid, and complex and all are found attached to mammalian glycoproteins (Alberts, 2002; Fukuda and Akama, 2003). These all share a common core structure that contains two GlcNac and three mannose, but differ in their outer branches. Biosynthesis of N-glycans includes “en bloc” transfer of oligosaccharides made of glucose, mannose and GlcNac utilizing several different enzymes to complete the process. For example, N-acetylgalcosaminytransferase I (GlcNAc-TI) is essential for the conversion of high mannose type N-glycans to hybrid type N-glycans. Mice lacking GlcNAc-TI die at embryonic day 10 due to inhibition of development (Ioffe and Stanley,
A-mannosidase II (MX) acts by removing two mannosyl residues from high mannose type N-glycans (Akama and Fukuda, 2006). Mice lacking MX show a great reduction in the level of GlcNAc-terminated N-glycans, and the spermatogenic cells of MX null mice fail to adhere to Sertoli cells leading to the failure of spermatogenesis (Akama et al., 2002).

BSG is a transmembrane glycoprotein rich in N-glycans (Tang et al., 2004). My results showed that GlcNAc terminated carbohydrates were greatly decreased in testes of Bsg KO mice. In addition, lectin blotting clearly showed the presence of GlcNAc terminated carbohydrates corresponding with BSG protein. Thus BSG may act as an attachment molecule for GlcNAc-terminated N-glycans.

To determine whether BSG plays a role in adhesion of spermatogenic cells to Sertoli cells, I then performed *in vitro* adhesion assays with SF7 cells and GC-2 cells transfected with *Bsg* siRNA. I found that knockdown *Bsg* in GC-2 cells caused a marked reduction in adhesion of GC-2 and SF7 cells. This failure of adhesion appears to be due to a defect in spermatogenic cells, not in Sertoli cells, which is consistent with previous reports (Akama et al., 2002). My findings support that BSG expressed on the surface of spermatogenic cells is crucial for germ cell adhesion to Sertoli cells.

Oligosaccharide 310.11, a GlcNAc-terminated N-glycan, has been shown to play an essential role in Sertoli-germ cell interactions (Akama et al., 2002). Pretreatment of Sertoli cells with this oligosaccharide dramatically blocked the binding between Sertoli
cells and germ cells. Expression of oligosaccharide 310.11 in MX KO testes showed a ~50% reduction compared to that in WT testes and led to failure of spermatogenesis. The phenotype of MX KO males included profound infertility with a great reduction in spermatozoa in the caudal ductus epididymis (Akama et al., 2002). My findings showed a marked reduction in the expression of GlcNAc-terminated carbohydrates in Bsg KO seminiferous tubules compared to the WT. However, the phenotype of the Bsg KO male is one of complete sterility with no mature spermatozoa found in the caudal ductus epididymis (Igakura et al., 1998) and with more severe defects in spermatogenesis than those observed in the MX KO males. This suggests that BSG probably regulates the expression of additional genes that are important for spermatogenesis.

Testes of Bsg KO mice also exhibited multinucleated giant cells. These multinucleated giant cells may form due to the failure of germ cells to lose their intercellular bridges. Similar multinucleated giant cells have been observed in the testes of MX KO mice (Akama et al., 2002), cAMP responsive element modulator (Crem) KO mice (Nantel et al., 1996) and Bcl-w KO mice (Print et al., 1998), which also show elevated apoptosis of male germ cells similar to Bsg KO males. CREM is a nuclear target of the adenylyl cyclase pathway. It binds to cAMP response elements, modulates the transcription of cAMP responsive genes and regulates gene expression (Lalli et al., 1996). In human testes CREM expression is restricted to the nuclei of round spermatids during spermatogenic stages I–III of seminiferous tubule differentiation. In the mouse, CREM is mainly expressed at stages VII-VIII and overall transcription stops at approximately stage IX (Sassone-Corsi, 1998; Weinbauer et al., 1998). The phenotype of the Crem
KO mouse is very similar to the Bsg KO mouse (Nantel et al., 1996). For example, late spermatids are completely absent and there is a significant increase in apoptotic germ cells. BCL-W is a pro-survival protein belonging to the BCL2 family (Print et al., 1998). Bcl-w KO mice also show a marked reduction in the number of elongating spermatids. Moreover, no mature spermatozoa are found in the testes of Bcl-w KO mice. Understanding the mechanism of action of both CREM and BCL-W in the testis would definitely provide insights into the functions of BSG during spermatogenesis.

In conclusion, my studies found that spermatocytes lacking BSG expression showed normal homologous chromosome synapsis and progression to the metaphase of first meiosis. However, spermatogenesis in Bsg KO males was arrested at the round spermatid stage. The loss of BSG in Bsg KO testes led to greatly reduced expression of CDH2 in the basal compartment of the seminiferous tubule and to disruption of the integrity of the BTB. Furthermore, BSG also appears to serve as an important attachment molecule between Sertoli cells and germ cells since BSG is a major N-glycans-containing glycoprotein in the testis. Loss of BSG significantly reduced adhesion between GC-2 and SF7 cells. Thus I believe that loss of BSG significantly impairs interactions between gametes and Sertoli cells. This is the reason for the large increase in the number of spermatocytes undergoing apoptosis in Bsg KO testes resulting in azoospermia. My results provide a direction for future studies of the interaction between Sertoli cells and germ cells. Elucidation of the specific functions of BSG during spermatogenesis may lead to the development of new therapeutic targets for reversing azoospermia.
Figure 5.1. Abnormalities in mouse testes due to loss of BSG expression. (A) Testes from WT and Bsg KO mice. Scale bar, 1mm. (B) The testis to body weight ratio of the KO mice was significantly less than that of the WT mice. Data are means ± SEM and the star indicates statistical differences (P< 0.05). (C) The testes from Bsg KO mice exhibit multinucleated giant cells (pachytene spermatocytes, arrowhead; round spermatids, arrow). Scale bar, 20 µm. (D) Abnormal chromatin patterns in the pachytene spermatocytes of Bsg KO testes. Scale bar, 20 µm. (E) Increased numbers of spermatocytes with pyknotic nuclei. Scale bar, 20 µm.
Figure 5.1 (cont’d). (F) TUNEL assay of WT and Bsg KO testes detected marked increases in apoptosis in Bsg KO testes. Apoptotic cells in the testes are stained brown in color. The rectangles located in the right-bottom of the images show enlarged views of the highlighted areas in each panel. Scale bar, 100 µm; scale bar (insert), 20 µm. (G) The diameter of both seminiferous tubule and seminiferous tubular lumen in Bsg KO testes were smaller than that of WT testes. Data are means ± SEM and the star indicates statistical differences (P< 0.05).
Figure 5.2. Surface-spread chromatin preparations documenting normal chromosome pairing, recombination and chromatin remodeling in WT and Bsg KO spermatocytes. (A-C) Immunofluorescence with antibodies to SYCP1 or SYCP3 in WT spermatocytes. (G-I) Immunofluorescence with antibodies to SYCP1 or SYCP3 in Bsg KO spermatocytes. Autosomal homologues were fully synapsed in terms of the labeling patterns of SYCP1 and SYCP3, a central element and a lateral element of the SC, respectively; and the merged panel identified the non-homologous X and Y (C, I, arrowhead).
Figure 5.2 (cont’d) (D–F) Immunofluorescence with antibodies to γH2AX or H1T in WT spermatocytes. (J–L) Immunofluorescence with antibodies to γH2AX or H1t in Bsg KO spermatocytes. The X and Y chromosomes were sequestered in a chromatin domain which was labeled intensely with antibody recognizing γH2AX (D, J). The antibody to H1T protein is a marker for spermatocytes that have reached or progressed beyond the mid-pachytene stage. SYCP1 labeling disappears, when chromosomes enter the MI division phase, and the SYCP3 labeling was seen at the centromeric ends of the chromosomes (F, L). There was no difference in localization of these markers between WT and Bsg KO spermatocytes.
Figure 5.3. Localization and quantitation of SP-10 protein in testes of WT and Bsg KO mice. (A) Immunohistochemistry for the acrosome marker SP-10 in WT and (B) Bsg KO testis sections. (C) Total proteins were isolated from whole testes and subjected to immunoblotting. (D) Bar graphs summarizing the densitometric analysis of the immunoblotting. Bars represent the mean ± SEM of three independent mice with the data normalized to the loading control, GAPDH. The rectangles located in the right-bottom corners of histological images show enlarged views of the highlighted areas in each panel. The brown color represents localization of protein. Scale bar, 100 µm; scale bar (enlargement), 20 µm.
Figure 5.4. Abnormalities in testes of aged Bsg KO mice. (A) PAS staining in aged WT, (B) young Bsg KO and (C) aged Bsg KO testis sections. Numerous vacuoles were formed within the seminiferous epithelium of aged Bsg KOs. Scale bar, 20 µm. (D) Loss of spermatogonia and failure of spermatogenesis in the seminiferous epithelium of aged Bsg KOs. Scale bar, 20 µm. (E) Sertoli cells (arrowhead) were observed clumped in the lumen of Bsg KO mice. Scale bar, 20 µm. (F) Abundant degenerating, sloughed germ cells were found in the lumen of efferent ductules in Bsg KO mice (arrowhead) in contrast to WT (F). Scale bar, 100 µm; scale bar (insert), 20 µm. The rectangles located in the right-bottom corner of images are the enlarged views of the highlighted areas in each panel.
Figure 5.5. **BSG immunoreactivity in mouse testes.** (A) BSG immunoreactivity was observed in every type of germ cell and in Sertoli cells. Scale bar, 50 µm. (B) Enlargement of insert of (A). Arrows show immunostaining of Sertoli-germ cell membranes. A, spermatogonium; P, pachytene spermatocyte; R, round spermatid; E, elongated spermatid; S, Sertoli cell nucleus. The brown color represents localization of BSG protein. Scale bar, 10 µm. (C) BSG immunoreactivity was observed in type A spermatagonia at 7 days of age. The red color represents the localization of Tra 98 in germ cells. The green color represents localization of BSG. Nuclei were stained with DAPI (blue). Scale bar, 10 µm. (D) There was no BSG immunoreactivity in the Bsg KO testis. Scale bar, 10 µm.
Figure 5.6. Integrity of the BTB and localization of BTB related adhesion molecules CXADR, CX43, CLDN11 and CDH2 in the testes of WT and Bsg KO mice. A biotin tracer was injected into the testicular interstitial space of both WT (A, B) and Bsg KO mice (H, I). Panels (B, I): The merged images of biotin tracer and DAPI. The biotin tracer penetrated around the early pachytene spermatocytes in compartment of Bsg KO seminiferous tubules (H, I, arrowhead). Panels (C, D): Immunohistochemistry with antibodies to CXADR or CX43 in WT testes. Panels (J, K): Immunohistochemistry with antibodies to CXADR or CX43 in Bsg KO testes. There was no difference in expression of CXADR and CX43 between WT and Bsg KO testes.
Figure 5.6 (cont'd) Panels (E, F): Immunohistochemistry with antibodies to CLDN11 or CDH2 in WT testes. Panels (L, M): Immunohistochemistry with antibodies to CLDN11 or CDH2 in Bsg KO testes. There was no difference in expression of CLDN11 between WT and Bsg KO testes. However, the expression of CDH2 was greatly reduced at the site of the BTB of Bsg KO mice. Panels (G, N): Sections were incubated with non-specific IgG as a negative control and there was no any staining. The brown color represents the localization of each protein, while green color represents the localization of biotin tracer. Scale bar, 20 µm
Figure 5.7. Localizations of GlcNAc terminated N-glycans in the testes of WT and Bsg KO mice. Localization of GlcNAc-terminated N-glycans in WT testes (A) and in Bsg KO testes (B). GSA II was strongly expressed in all phases of WT spermatogenic cells except spermatogonia (arrowhead). This lectin staining, however; was greatly decreased in the Bsg KO testes. (C) Sections incubated with GSA II that was pretreated with N-acetylglucosamine as a negative control did not show any positive staining. The rectangles located in the bottom-right corner of images are the enlarged views of the highlighted areas in each panel. The brown color represents localization of GlcNAc-terminated N-glycans. Scale bar, 100 µm; scale bar (insert), 20 µm.
Figure 5.8. GlcNAc-terminated N-glycans are one of the carbohydrates linked to BSG. BSG protein was immunoprecipitated from mouse testes lysates with BSG antibody. Immunoblotting with BSG antibody indicated that the protein immunoprecipitated from mouse testes lysates was BSG. No BSG signal was observed from the IgG control. After stripping the blot, lectin blotting with GSA II was performed to show the presence of GlcNAc terminated N-glycans.
Figure 5.9. Adhesion of GC-2 spermatocyte cells transfected with Bsg siRNA to SF7 Sertoli cells. (A) Loss of BSG protein expressions in Bsg siRNA transfected cells. Protein lysates for BSG were isolated from both Bsg siRNA treated and control GC-2 cells and subjected to immunoblotting. GAPDH served as a loading control. (B) Adhesion between siRNA treated GC-2 cells and SF7 cells was significantly decreased in comparison to the control group. Bars represent the fold change ± SEM of three independent experiments and the asterisk indicates the statistical difference (P< 0.05).
References


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Chapter Six
Summary of Results and Future Directions

Implantation is the process by which the free-floating blastocyst attaches to the uterine endometrium, invades into the stroma and establishes the placenta. It is the result of a complex series of reciprocal interactions between the conceptus and a receptive uterus. There are two main processes during implantation: uterine receptivity/ conceptus invasion and the decidual cell reaction (decidualization). Basigin (BSG) is essential for successful implantation because $Bsg$ null mutant embryos fail to implant and transfer of wild-type blastocysts to $Bsg$ null mutant mice results in a low birth rate. Moreover, BSG plays a role in regulating the timing of decidualization in mice. Therefore, in this study I examined the expression, regulation and function of BSG during early pregnancy using in vitro and in vivo experimental models. I hypothesized that: 1) BSG is essential for sensitizing the uterine luminal epithelium in mice during early pregnancy. 2) BSG is necessary for normal decidualization of human uterine stromal cells (HESCs).

Furthermore, BSG is also involved in the process of spermatogenesis. Spermatogenesis is the process of producing sperm (haploid) from initially undifferentiated germ cells (diploid). During spermatogenesis, there are a group of somatic cells named Sertoli cells that nurse and communicate with all the different types of germ cells within the seminiferous tubules, via direct contact. In the $Bsg$ KO testes, there is a severe defect in spermatogenesis and no mature sperm are found in the testis or the epididymis. Therefore, in this study I examined the expression, regulation and function of BSG during spermatogenesis using in vitro and in vivo experimental models.
I hypothesized that BSG is essential for the interactions between gametes and Sertoli cells during spermatogenesis.

Using *in vitro* and *in vivo* model systems, I was able to confirm all three hypotheses. In summary, the main findings and future directions of my research studies are:

**BSG is essential for sensitizing the uterine luminal epithelium in mice during early pregnancy**

BSG protein was expressed in the uterine epithelium at estrus in βERKO mice but not in αERKO mice. However, a higher level of Bsg mRNA was observed in the uteri of αERKO mice as compared with wild type (WT) and βERKO mice. These apparently contradictory observations suggest that the signaling mediated by ESR1 must be necessary for BSG translation, instead of mRNA transcription. In the mouse, estrogen alone induces the proliferation of both luminal and glandular epithelial cells during early pregnancy. On day 1 of pregnancy, the expression levels of ERα and a well-known estrogen responsive gene, MUC1, appeared to be normal in the uteri of Bsg KO females. This suggested that the circulating estradiol levels in Bsg KO mice are normal.

I examined proliferation in uterine epithelial cells and found that, in WT mice, uterine epithelial cells were highly proliferative as measured by expression of Ki67 in both the luminal and glandular epithelial cells. In contrast, Ki67 expression was significantly decreased in the epithelial cells of Bsg KO uteri. My findings suggest that expression of BSG protein in the uterus requires estrogen acting through ESR1, but not ESR2. Moreover, estrogen may regulate the proliferation of both luminal and glandular epithelial cells in the uterus during early pregnancy through a pathway involving BSG.
My results showed there was no difference in the expression of MUC1 in the uteri of Bsg KO and WT mice. However, immunochemistry can only provide an estimate of the levels of expression of estrogen regulated proteins and may not be able to distinguish subtle differences. Therefore, it is necessary to determine the endogenous estrogen levels in Bsg KO mice on day 1 of gestation by radioimmunoassay. Moreover, conditional gene knockout is a very powerful technique to knock out a specific gene from specific tissues. It will be really helpful to understand the function of BSG during early pregnancy if I can knock out Bsg specifically from uterine luminal epithelium.

**BSG is necessary for normal decidualization of human uterine stromal cells**

HESCs were transfected with small interfering RNAs targeting BSG gene expression. Expression of the decidualization markers Insulin-like growth factor-binding protein 1 (IGFBP1) and Prolactin (PRL) was significantly inhibited in cells with down regulated BSG expression. These results indicated that BSG is necessary for normal decidualization of HESCs. Silencing of BSG in HESCs also impaired expression of several MMPs. Microarray analysis revealed that both WNT4 and FOXO1 and its downstream targets are under the regulation of BSG during decidualization in HESCs. These results have provided new insights into the molecular pathways that regulate decidualization of human uterine stromal cells. Understanding the role of BSG during decidualization may help to explain the defects in decidualization-associated reproductive disorders of women.
My microarray analysis revealed that both WNT4 and FOXO1 and its downstream targets are under the regulation of BSG during decidualization in HESCs. It will be really useful if the protein levels of WNT4, active β-catenin, FOXO1 and its downstream targets are studied further by immunoblotting and immunocytochemistry. Moreover, adenovirus-mediated overexpression of BSG in HESCs could allow us to observe the effects of excess BSG expression on decidualization.

**Sterility in Basigin Null Mutant Male Mice May Be Due to Impaired Interactions Between Gametes and Sertoli Cells**

The *Bsg* KO testis lacks elongated spermatids and mature spermatozoa, a phenotype similar to that of alpha-mannosidase IIx (MX) KO mice. MX regulates formation of N-acetylglucosamine (GlcNAc) terminated N-glycans that participate in germ cell-Sertoli cell adhesion. Results showed that *Bsg* KO spermatocytes displayed normal homologous chromosome synapsis and progression to the midpachytene stage. Both meiosis I (MI) and meiosis II (MII) spermatocytes were detected in the KO preparations. However, expression of the acrosome marker SP-10 was extremely low in germ cells of *Bsg* KO mice indicating that spermatogenesis in *Bsg* KO mice was arrested at the round spermatid stages. I observed a large increase in the number of germ cells undergoing apoptosis in *Bsg* KO testes. Using lectin blotting, I determined that GlcNAc terminated N-glycans are linked to BSG. GlcNAc terminated N-glycans were significantly reduced in *Bsg* KO testes. These observations indicate that BSG may act as a germ cell-Sertoli cell attachment molecule. Loss of BSG significantly reduced adhesion between GC-2 and SF7 cells. Moreover, WT testes showed strong expression of N-cadherin (CDH2)
while expression was greatly reduced in the testes of Bsg KO mice. In addition, the integrity of the blood-testis barrier (BTB) was compromised and flow of fluid from the testis was reduced in Bsg KO testes. In conclusion, although Bsg KO spermatogonia can undergo normal progression to the spermatocyte stage, BSG-mediated germ cell-Sertoli cell adhesion via GlcNAc terminated N-glycans appears to be necessary for integrity of the BTB and spermatocyte progression to mature spermatozoa.

My results showed that Bsg KO spermatocytes enter MI and MII metaphase. However, it is still unknown whether the number of cells which enter MI and MII are normal or less than those of WT males. Therefore, it is important to repeat the DNA staining to further validate these findings. Moreover, the testes from Bsg KO mice exhibited multinucleated giant cells. Similar multinucleated giant cells have been observed in the testes of MX KO mice. CREM KO mice and Bcl-w KO mice also show elevated apoptosis of male germ cells similar to my Bsg KO males. I would like to determine the expression pattern and localization of Bcl-w in Bsg KO mice. This would definitely give us an insight into the function of Bsg during spermatogenesis.

In conclusion, the findings of my dissertation have made a substantial contribution to understanding the function of BSG in implantation/decidualization and spermatogenesis. Further studies with this protein would help elucidate its specific role in both these processes.
Chapter Seven
Materials and Methods

Materials

1X Carbo-free blocking buffer (Vector Laboratories, Burlingame, CA)

8-bromo-cAMP (Sigma, St. Louis, MO)

17β-estradiol (Sigma, St. Louis, MO)

Agilent Human arrays (Agilent Technologies, Santa Clara, CA)

ApopTag Plus Peroxidase In Situ Apoptosis Kit (Chemicon-Millipore, Billerica, MA)

Assays-on-Demand™ Gene Expression Assay (Life Technologies, Carlsbad, CA)

Biotinylated *Griffonia simplicifolia* agglutinin (GSA) II (Vector Laboratories, Burlingame, CA)

Bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA)

Citric acid (Fisher Scientific, Pittsburgh, PA)

Collagenase (Sigma, St. Louis, MO)

Dulbecco’s Modified Eagle’s medium (DMEM) /F12 (Life Technologies, Carlsbad, CA)

DMEM (ATCC, Manassas, VA)

EZ-Link Sulfo-NHS-LC-Biotin (Fisher Scientific, Pittsburgh, PA)

Fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA)

Fluorescein Isothiocyanate (FITC) conjugated streptavidin (Life Technologies, Carlsbad, CA)

High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA)

HRP donkey anti-goat IgG antibody (abcam)

Hydrogen peroxide (Fisher Scientific, Pittsburgh, PA)
Immobilon-P membranes (PVDF) (Millipore, Billerica, MA)
Immunopure Avidin Hoseradish Peroxidase Conjugated (avidin-HRP) (Fisher Scientific, Pittsburgh, PA)
Isoflurane (Attane, Minrad, Bethlehem, PA)
Isotype control IgG (Vector Laboratories, Burlingame, CA)
L-glutamine (Cellgro, Manassas, VA)
Mayer's Hematoxilin solution (Sigma, St. Louis, MO)
Metal-3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO)
Modified Davidson’s solution (Electron Microscopy Sciences, Hatfield, PA)
Multispot microscope slides (Shandon, Pittsburgh, PA)
N-acetylglucosamine (Sigma, St. Louis, MO)
OPTI-MEM I reduced-serum medium (Life Technologies, Carlsbad, CA)
Paraffin (Fisher Scientific, Pittsburgh, PA)
Paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA)
Penicillin-streptomycin (Cellgro, Manassas, VA)
Pierce® BCA protein assay kit (Fisher Scientific, Pittsburgh, PA)
Poly-L-lysine-coated slides (Fisher Scientific, Pittsburgh, PA)
Potassium chloride (Fisher Scientific, Pittsburgh, PA)
Potassium phosphate (Sigma, St. Louis, MO)
Precise Protein Gels (Fisher Scientific, Pittsburgh, PA)
Precision Plus Protein All Blue Standards (BioRad, Hercules, CA)
Progesterone (Sigma, St. Louis, MO)
Protein A/G Plus-Agarose Immunoprecipitation Reagent (Santa Cruz Biotechnology, Santa Cruz, CA)

Restrore™ western blot stripping buffer (Fisher Scientific, Pittsburgh, PA)

REDExtract-N-Amp (Sigma, St. Louis, MO)

RNeasy Mini Kit (Qiagen, Valencia, CA)

Scintiverse scintillation liquid (Fisher Scientific, Pittsburgh, PA)

Secondary antibody anti-goat IgG and conjugated with CY3 (Jackson ImmunoResearch, West Grove, PA)

Sesame oil (Sigma, St. Louis, MO)

Silencer select negative control #1 (Life Technologies, Carlsbad, CA)

Silencer select siRNA (Life Technologies, Carlsbad, CA)

SiLentFect™ Lipid (Bio-Rad, Hercules, CA)

Sodium chloride (Sigma, St. Louis, MO)

Sodium citrate (Fisher Scientific, Pittsburgh, PA)

Sodium phosphate dibasic heptahydrate (Fisher Scientific, Pittsburgh, PA)

SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific, Pittsburgh, PA)

TaqMan Universal PCR Master Mix (Life Technologies, Carlsbad, CA)

Tritiated thymidine ([³H] thymidine) (PerkinElmer, Waltham, MA)

Triton X-100 (Sigma, St. Louis, MO)

Trypsin (Sigma, St. Louis, MO)

Trypsin EDTA (Cellgro, Manassas, VA)

TRIzol® Reagent (Life Technologies, Carlsbad, CA)

Tween 20 (Fisher Scientific, Pittsburgh, PA)
Animals used in this research were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Illinois. The wild-type C57BL6/J male mice were obtained from Jackson Laboratory (Bar Harbor, ME) and were housed under temperature and light controlled conditions (12 h light: 12 h dark) with free access to food and water. C57BL6/J heterozygous (Bsg +/-) mice were a kind gift from Dr. Takashi Muramatsu, Department of Biochemistry, Nagoya University School of Medicine, Japan. Heterozygote breeding was carried out in the animal facility of the University of Illinois. At 3 weeks of age, offspring were anesthetized by isoflurane inhalation (Attane; Minrad, Bethlehem, PA) and tail snips were collected for genotyping. DNA was extracted from the snips using REDExtract-N-Amp (Sigma, St. Louis, MO), and genotypes determined by PCR using primers for Bsg and neomycin (Igakura et al., 1998). Offspring with the Bsg -/- phenotype were housed under the same conditions as those for the WT mice. Female WT and Bsg-null mice were ovariectomized at 10 to 12 weeks of age and rested for 2 weeks. These mice were then treated with a single dose of 17β-estradiol (E) (200 ng in sesame oil) by intraperitoneal (i.p.) injection and uteri were harvested at 24 hours after injection.

The generation of mice null for the Esr1 (ERα) or Esr2 (ERβ) genes was in a C57BL6/J background and has been described previously (Krege et al., 1998; Lubahn et al.,
All mice used for this study were 40-60-days old and provided by Dr. Kenneth S. Korach, Laboratory of Reproductive and Developmental Toxicology, National Institutes of Health, Research Triangle Park, NC.

Histological sections of uteri from $\text{C/EBP}\beta$ null mice were provided by Dr. Milan Bagchi of University of Illinois. Briefly, female WT and $\text{C/EBP}\beta$-null mice of the 129Sv background were ovariectomized at 10 to 11 weeks of age and rested for 2 weeks. These mice were then treated with a single dose of 17β-estradiol (E) (250 ng in sesame oil) by intraperitoneal (i.p.) injection and uteri were harvested at various times after injection.

**Tissue collection and cell culture**

**Human decidual tissues**

Human uterine tissues were obtained from patients with elective terminations of first-trimester pregnancies (n=3). Human samples were collected and immediately processed for immunohistochemical analysis. All procedures were under approved protocols at John Stroger Jr. Hospital of Cook County and the Institutional Review Board of the University of Illinois at Chicago.

**Human endometrial stromal cell culture**

Telomerase-immortalized human endometrial stromal cells (HESCs) (Krikun et al., 2004) were cultured in DMEM/F12 supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin in a 5% CO$_2$ humidified incubators at 37°C.
**Mouse spermatocyte and Sertoli cell culture**

GC-2 cells (Hofmann et al., 1995) are an immortalized spermatocyte cell line and were purchased from the American Type Culture Collection (CRL-2196) (ATCC, Manassas, VA). Cells were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin in a 5% CO₂ humidified incubators at 37°C.

SF7 cells (Hofmann et al., 1992) are an immortalized Sertoli cell line and were a gift from Dr. Hofmann at University of Illinois. Cells were cultured in DMEM/F12 supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin in a 5% CO₂ humidified incubators at 37°C.

**Tissue collection**

Both female (6-8 weeks of age) and male (7 days, 12–24 weeks or 15-17 months of age) mice were euthanized with carbon dioxide. Uteri, ovaries and testes were collected immediately. Tissues for histology were fixed in modified Davidson's solution or 4% paraformaldehyde in PBS overnight. The next day, tissues were rinsed with ethanol and paraformaldehyde was replaced with 70% ethanol. Specimens were then processed for paraffin embedding using routine procedures (Table 6.1). Tissues for Western and lectin blotting analyses were snap-frozen in liquid nitrogen and stored at -80°C until use.

**Germ cell isolation and spermatocyte enrichment**

Testes were removed from both WT and Bsg KO mice, detunicated and digested in 0.5 mg/ml collagenase (Sigma, St. Louis, MO) in Krebs-Ringer bicarbonate solution (KRB)
[120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 1.3 mM CaCl₂, 11 mM glucose, 1 X essential amino acids, 1 X nonessential amino acids] at 32°C for 20 min, followed by digestion in 0.5 mg/ml trypsin (Sigma) containing 20 mg/ml DNase I in KRB at 32°C for 13 min. After digestion the cell suspension was filtered through an 80-µm mesh filter and washed three times in KRB. Germ cells were then processed as described below.

**Small interfering RNA (siRNA) transfection for HESC**

SiRNAs corresponding to human BSG (sense: CGUAGAUCUCUCAUUCAUCAAtt; antisense: UGUAUGAUGGAAUCUACGgg) were purchased from Ambion. Silencer select negative control #1 was used as a negative control under the same conditions. The BSG siRNA or negative control #1 were transfected into HESCs following the protocol of siLentFect™ Lipid. Briefly, 3 µl of siLentFect Lipid transfection reagent were mixed with 5 nM of siRNA or negative control #1 to form complexes and were dispersed into 6-well cell culture plates at 37°C. After complete knocking down BSG in HESCs (72 hours after adding siRNA), HESCs were treated with media containing estrogen, progesterone and cAMP to induce decidualization (0.5 mM 8-bromo-cAMP, 1 µM progesterone and 10 nM 17β-estradiol for 0-8 days (Li et al., 2007)). BSG siRNA and negative control #1 were also added every four days (day 0, 4). Total RNA and protein were harvested at each time point (day 0, 2, 4, 6, 8) for either Quantitative RT-PC or immunoblotting.
Small interfering RNA (siRNA) transfection and in vitro assay for germ cell-Sertoli cell adhesion

Methods for mouse spermatogenic cells (GC-2) and Sertoli cell (SF7) adhesion assays (Akama et al., 2002) were performed as previously described with the following modifications. SiRNAs corresponding to mouse Bsg (sense: GGCUGUACAUUGAGCAGAAtt; antisense: UUCUGCUAAUGUACAGCCt) were purchased from ambion (Life technologies, Grand Island, NY). The siRNA was transfected into the GC-2 cells following the protocol of siLentFect™ Lipid (Bio-Rad, Hercules, CA). Briefly, 3 µl of siLentFect Lipid transfection reagent was mixed with 10 nM of siRNA to form complexes and dispersed into 6-well cell culture plates at 37°C for 48 hours. Silencer select negative control #1 (Life technologies) was used as a negative control under the same conditions.

The GC-2 cells were then labeled with [³H] Thymidine (2 µCi/ml) (Perkin Elmer, Waltham, MA) at 37°C for another 24 hours. 4.2X10⁵ [³H]-labeled GC-2 cells were added to the SF7 cells and incubated at 37°C for 4 hours. After washing unbound cells with PBS, radioactivity remaining on the SF7 cells was counted in a scintillation counter.

Cell Proliferation

HESCs were seeded at a density of 2.5 x 10⁴ cells/well in 6-well plates and were cultured in the culture medium containing with DMEM/F12, 10% FBS, 1% L-glutamine and 1% Penicillin–Streptomycin for 24 hours. HESCs were then transfected with either
BSG siRNA or negative control #1. After 48 hours, the siRNA and negative control #1 were removed and cells were cultured in the culture medium for another 24 hours. Cells were then harvested and the number of cells was counted.

For tritiated thymidine incorporation arrays, HESCs were transfected with either BSG siRNA or negative control #1 for 48 hours. HESCs were then labeled with $[\text{^3H}]$ Thymidine (2 µCi/ml) (Perkin Elmer, Waltham, MA) at 37°C for another 24 hours. Cells were then harvested and counted in a scintillation counter.

**Microarray analysis**

Total RNA was purified on day 6 of the decidualization following the protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA) and then utilized for microarray analysis, using the Agilent Human arrays (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed in the University of Illinois at Urbana-Champaign Biotechnology Center. Briefly, Microarray data preprocessing and statistical analyses were done in R using the limma package. Median foreground and median background values from the 4 arrays were read into R and any spots that had been manually flagged (~100 values) were given a weight of zero. The background values were ignored because investigations showed that trying to use them to adjust for background fluorescence added more noise to the data; background was low and even for all arrays, so no background correction was done.
The M-values (log2 ratio of Cy5/Cy3 for each spot) for each array were first normalized within each array using the loess method and then normalized between arrays using the scale method. Agilent's Human Gene Expression 4x44K v2 Microarray interrogates 27,958 genes using 33,128 probes spotted one time (1X) and 999 probes spotted ten times (10X) each. After normalizations and removal of the positive and negative control probes, the data were split in two and separate statistical models were fit for the 1X probes and the 10X probes. The models were nearly identical linear models from the limma package, both assessed the difference between SI and NC and controlled for overall dye effect, but the model for the 10X probes additionally adjusted for the replicate spots per probe. The results for all 34,127 probes were combined back together and the raw p-values were adjusted using the False Discovery Rate method separately for each comparison.

Tissue and cell lysate for immunoblotting

Frozen tissues were ground in liquid nitrogen and solubilized in modified RIPA buffer in the presence of protease inhibitors (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS and proteinase inhibitor).

Total cell lysate from each cell culture that had received various treatments in specific experiments were harvested by scraping cells into hot (95 °C) 1x Laemmli Sample Buffer (LSB) (10% glycerol, 62.5 mM Tris pH 6.8, and 2% Sodium Dodecyl Sulfate, and 5 mM sodium orthovanadate). Harvested cell lysates were then collected in eppendorf tubes, sonicated for 10 seconds, and then spun at 14,000xg for 10 min at 4 °C to
remove nuclear and cell debris. The supernatant was then transferred to fresh eppendorf tubes for immediate use or storage at -20°C for future use.

**Immunoblotting analysis**

BCA protein assays were performed on cell lysates to determine protein concentrations. Fifteen to twenty micrograms of total protein were resolved by 10% SDS-PAGE. The gels were transferred to Immobilon-P membranes (PVDF), blocked in 5% non-fat dry milk in TBST (20 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20), and probed with primary antibody according to conditions specific for each primary antibody (Table 6.2) overnight at 4°C. The membranes were washed 3 times with TBST and incubated with the HRP-conjugated secondary antibody for 60 min at room temperature. After three 5 minutes washes in TBST, the SuperSignal West Pico Chemiluminescent Substrate was applied to membranes for 5 minutes before exposure to autoradiography films. The same membranes were stripped in Restore western blotting stripping buffer for 15 minutes at room temperature and re-probed with anti-GAPDH antibody as a loading control.

**Densitometric analysis of immunoblotting**

Films developed after exposure to chemiluminescence were scanned, and images were analyzed using the ImageJ software from NIH (available at [http://rsbweb.nih.gov/ij/download.html](http://rsbweb.nih.gov/ij/download.html)). Measurements of BSG bands were normalized for loading differences using measurements of GAPDH. Graphs represent means and SEM.
Histology, immunohistochemistry and immunofluorescence

Paraffin embedded tissues were sectioned at 4-5 µm and mounted on poly-L-lysine-coated slides. After deparaffinization in xylene, the sections were rehydrated in decreasing concentrations of ethanol (twice 5 min in 100% ethanol, 2 min in 100% ethanol, 2 min in 95% ethanol, 80% ethanol, 70% ethanol) and then 2 min wash in water. Sections were stained using the periodic acid-Schiff (PAS) reaction (Cerri and Sasso-Cerri, 2003) and Mayer’s hematoxylin for histological observation.

Sections for immunostaining were boiled in 10 mM citrate buffer (Mix 18 ml of 0.1 M citric acid, 82 ml of sodium citrate and 900 ml of H2O, pH 6.0) for 30 minutes to promote antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide for 15 minutes. After blocking with 2% normal serum in PBS/Tween 20 (PBS/T) for 60 minutes, sections were incubated at 4ºC overnight with primary antibody according to conditions specific for each primary antibody (Table 6.2). Isotype control IgG was applied as a negative control. After washing in PBS/T, sections were incubated with secondary antibody either conjugated with CY3 at 1:1000 with PBS/T or biotin diluted at 1:200 with PBS/T for 60 minutes at room temperature. Sections for immunohistochemistry were then incubated in ABC solution or 30 minutes, reacted with metal-3,3’-diaminobenzidine (DAB) for minutes, and counterstained with Mayer’s hematoxylin for 1 minute before washing sections in tap water, dehydration, clearing and mounting. Sections for immunofluorescence were then mounted using Vectashield.
Cytological analyses

For immunofluorescence analysis, cells were collected by centrifugation, surface-spread in wells of multispot microscope slides and fixed following the procedure previously described (Cobb et al., 1999b; Cobb et al., 1997). Prior to antibody labeling, slides were washed three times in washing/blocking buffer (0.3% BSA, 1% goat serum in phosphate-buffered saline, pH 7.4); the second wash included 0.05% Triton-X 100. After draining, the slides were incubated with primary antibodies. Antibodies and dilution used were: rabbit anti-SYCP1 and SYCP3 used at 1:100 dilution; guinea pig anti-H1T (Cobb et al., 1999a) at 1:500; rabbit anti-γH2AX at 1:200; Secondary antibodies against rabbit, rat or mouse IgG and conjugated with Alexa 594 or 488 were used at 1:500 dilution. Images were acquired with a Leica DMRXE epifluorescence microscope equipped with a 100X plan-neofluar oil-immersion objective lens and a triple filter (set no. 61000V2 BS&M, Chroma Technology, Rockingham, VT) for simultaneous visualization of green (Alexa 488), red (Alexa 594), yellow (Alexa 488 + Alexa 594) and blue (DAPI) fluorescence. The microscope was linked to a Micromax cooled CCD camera (RS Princeton Instrument) with a high-speed shutter driven by a Sutter Lambda 10-2 (Sutter Instrument) and Metamorph software (Universal Imaging Corporation) to capture the images.

RNA isolation and quantitative reverse transcription-PCR (qPCR)

Total RNA was extracted from mouse uteri, ovaries or HESCs using TRIzol™ according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit following the
Real-time PCR analyses were performed using TaqMan® Universal PCR Master Mix No AmpErase® UNG. Genes were amplified using 20 x Assays-on-Demand™ Gene Expression Assays purchased from Life Technologies. The following 20x Assays-on-Demand™ Gene Expression Assay primer-probe sets were used for my studies: Bsg (Mm00814798_m1), Lactoferrin (Ltf) (Hs00236877_m1), 18S (Hs99999901_s1), Prolactin (PRL) (Hs00168730_m1), Insulin-like growth factor-binding protein 1 (IGFBP1) (Hs00236877_m1), and POP4 (HS00198357_m1). Briefly, 4.5 µl of a 1:10 diluted cDNA sample was mixed with 5.5 µl of master mix (5µl of TaqMan Universal PCR Mix and 0.5 µl of 20X Assay on Demand) for a total volume of 10 µl per well in a MicroAmp optical 384-well reaction plate. Three experimental replicates were performed for each sample. qPCR amplification and quantitation was performed using the ABI 7900 sequence detection system for 40 cycles (95°C for 15 sec, 60°C for 1min). The comparative CT method (ΔΔCt) was used for quantification of gene expression. Relative fold differences in gene expression for all tested genes were normalized to the either 18S or POP4 endogenous control.

**Radioimmunoassay**

Blood samples were collected from WT and Bsg KO mice (n=3) and serum samples were stored at -80°C until the assays were performed. Testosterone levels were measured using a radioimmunoassay performed by the University of Virginia Core Ligand and Assay Laboratory. The limit of detection for this assay was 0.1 ng.
**Tunel staining**

Detection of DNA fragmentation of apoptotic cells was performed by using an ApopTag Plus Peroxidase In Situ Apoptosis Kit. Briefly, after deparaffinization and rehydration, sections were permeabilized by incubation with proteinase K (20 mg/ml) for 15 minutes. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide in PBS for 5 minutes. After a 10 second incubation in equilibration buffer, sections were incubated in the terminal deoxynucleotidyl transferase (TdT) labeling reaction mix for 1hr at 37ºC. After a 10-minute wash in stop/wash buffer, sections were incubated with antidigoxigenin and finally developed in DAB. For the negative controls, sections were processed without TdT enzyme in the labelling reaction mix. Sections were counterstained with 0.5% (w/v) methyl green for 10 min at room temperature.

**Barrier function assays**

The permeability of the BTB was assessed using the method developed by Chen et al. (Chen et al., 1997). Briefly, WT and BsgKO mice (12 weeks old) were anesthetized using ketamine/xylazine (2mg/0.2mg). Testes were exposed by a small incision through the scrotal layer. Next, a small opening in the tunica albuginea was gently created with fine forceps and 20ul of 10 mg/ml EZ-Link Sulfo-NHS-LC-Biotin were injected using a Hamilton syringe into the interstitium of one testis. The other testis was injected with 20ul of PBS as a control. After 30 min the animals were euthanized by cervical dislocation, and the testes were immediately removed and fixed in 4% paraformaldehyde. Testes were then processed for paraffin embedding. The slides were deparaffinized, rehydrated, boiled in 10 mM citrate buffer (pH 6.0) for 30 minutes
and incubated with Fluorescein Isothiocyanate (FITC) conjugated streptavidin for 60 minutes. Slides were then mounted with vectashield.

**Lectin histochemistry**

Tissue sections were deparaffinized in xylene three times for a total of 60 minutes. The sections were rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked in methanol containing 3% hydrogen peroxide for 20 minutes. After blocking with 1% BSA in PBS/T for 60 minutes, sections were incubated at 4°C overnight with 10µg/ml biotinylated *Griffonia simplicifolia* agglutinin (GSA) II. GSA II that was preincubated with N-acetylglucosamine was applied as a negative control. After washing in PBS/T, sections were incubated in ABC solution for 30 minutes, reacted with DAB for 3 minutes, and counterstained with hematoxylin.

**Lectin blotting analysis**

Membranes were blocking in 1X Carbo-free blocking buffer for an hour, and then incubated with 1µg/ml GSA II overnight at 4°C. Membranes were then incubated in 0.2µg/ml Immunopure Avidin Hoseradish Peroxidase Conjugated (avidin-HRP) for 1 hr. After three 5 minutes washes in TBST, the SuperSignal West Pico Chemiluminescent Substrate was applied to membranes for 5 minutes before exposure to autoradiography films.
**Immunoprecipitation**

Tissues were homogenized and proteins extracted using TNE buffer (1% NP 40, 10mM Tris pH 8, 1mM EDTA). 500 µg of protein were incubated with 2 µg of goat polyclonal antibody against mouse BSG or goat IgG for 60 minutes at 4ºC. Protein G plus-Agarose was added into each protein sample overnight at 4ºC. Immunoprecipitates were collected by centrifugation at 2500 rpm at 4ºC and washed with RIPA buffer. The protein was removed from the beads by boiling for 5 minutes in 1X LSB (2% SDS, 10% glycerol, 6.25mM Tris pH 6.8). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.

**Statistical analysis**

The samples for this study were prepared from at least replicates subjected to the same experimental treatments. Results are represented as mean ± SEM. Differences between groups were examined using Student’s t-test. Statistical significance was set as $P<0.05$. 
### Table 7.1. The procedure of tissue process

<table>
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<tr>
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Table 7.2. List of primary antibody

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<th>Dilution</th>
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<td>R&amp;D</td>
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</tr>
<tr>
<td>Rabbit polyclonal to mouse ESR1</td>
<td>1:200 (IHC)</td>
<td>Santa Cruz</td>
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