Female sterility in mice lacking the *basigin* gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily

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Abstract Basigin (Bsg) is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. Bsg knock-out mice exhibit infertility of both sexes. Based on limited results, defective implantation has been considered to be the cause of the female infertility. We demonstrate here that disruption of the *Bsg* gene produces the failure of female reproductive processes including not only implantation but also fertilization. Bsg mRNA expression in cumulus cells and basolateral localization of the Bsg protein in the endometrial epithelium further support the importance of Bsg in these processes.

Key words: Azoospermia; Fertilization; Gene targeting; Implantation; Matrix metalloprotease; Oocyte maturation

1. Introduction

Basigin (Bsg) is a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily with two Ig domains in its extracellular portion [1,2]. Chicken Bsg (HT7/neurothelin/5A11) has been implicated in construction of the blood-brain barrier, as it is specifically expressed in capillary endothelial cells in the brain [3–6]. It has also been implicated in the neuronal-glial interaction in the retina during development [7,8]. Human Bsg (M6/EMMPRIN) has been implicated in the activation of lymphocytes and the induction of matrix metalloproteases [9,10].

We generated Bsg knock-out mice. Bsg null mutants exhibit normal functions of the blood-brain barrier [11]. However, they show unexpected phenotypes, as follows. Many Bsg null mutant embryos are lost around the time of implantation [12]. Adult null mutants exhibit lowered sensitivity to irritating odors [11], and deficits in spatial learning and memory [13]. Moreover, both surviving male and female Bsg null mutants are infertile [12]. In the case of males, spermatogenesis is arrested at the prophase of the first meiosis, consequently the adult mice exhibit azoospermia [12]. In contrast to the clear cause of male infertility, the cause of female sterility has been obscure. In a previous study, it was suggested that the primary cause of female sterility may be the failure of implantation because most blastocysts transferred to the uteri of null mutants were lost in early pregnancy and Bsg mRNA expression was regionally elevated at the site of embryo apposition in the uterine endometrium [12]. In the present study, to verify the roles of Bsg in pregnancy and to determine the cause of their infertility, we have extended the previous studies by systematically examining female null mutants.

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Abbreviations: Bsg, basigin; Ig, immunoglobulin; hCG, human chorionic gonadotropin; BSA, bovine serum albumin; PBS, phosphate-buffered saline

2. Materials and methods

2.1. In vitro fertilization

Pregnant mare serum (5 IU) dissolved in saline was injected intraperitoneally into each female mouse at 16.00 h. Forty-eight hours later, 5 IU of human chorionic gonadotropin (hCG) was injected in the same way. At 14 h after injection of the hCG into the female mice, male mice were killed by cervical dislocation. Their epididymides were resected and punctured with a needle. A white drop of the sperm was introduced into a drop of Whittingham's medium containing 30 mg/ml of bovine serum albumin (BSA) covered by mineral oil, followed by incubation for 1 h at 37°C under 5% CO_2-95% air. At 16 h after the injection of hCG, the female mice were killed and their oviducts were resected. Oocytes covered with follicular cells were introduced into a drop of Whittingham's medium containing 30 mg/ml of BSA equilibrated under the same conditions as for sperm. Whittingham's medium containing sperm was added to a drop containing oocytes to give a final concentration of 200 sperm/μl. After 6 h incubation, the oocytes were transferred to M16 medium containing 4 mg/ml of BSA. The oocytes were incubated overnight and those exhibiting cell division were regarded as being fertilized.

2.2. Histological analyses

Mice were anesthetized with Nembutal, and then perfused with 10 ml of saline followed by 50 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). The tissues were removed and fixed in 4% paraformaldehyde in PBS at 4°C overnight. After embedding in paraffin, sections of 5 μm thickness were cut and placed on silane-coated slide glasses, and then subjected to staining or in situ hybridization.

Generation of anti-Bsg antibodies was performed as described previously [14]. For immunohistochemistry, a uterus was fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), followed by serial washing with 10, 15 and 20% sucrose in phosphate buffer. The specimen was embedded in OCT compound, and then frozen sections were cut and placed on lysine-coated slide glasses. The sections were incubated in Tris-HCl buffer (pH 7.4) containing anti-Bsg antibodies (dilution 1:50) overnight at 4°C. After thorough washing with PBS, the sections were processed with FITC-labeled anti-mouse IgG for 1 h at room temperature. The sections were washed with PBS and then examined under a fluorescence microscope.

In situ hybridization for Bsg expression was performed as described previously [14].

2.3. Northern blot analysis

Northern blot analysis was performed as described previously [15]. Briefly, total RNA was extracted from freshly removed tissues by the method described previously [16]. 10 μg of each RNA was separated

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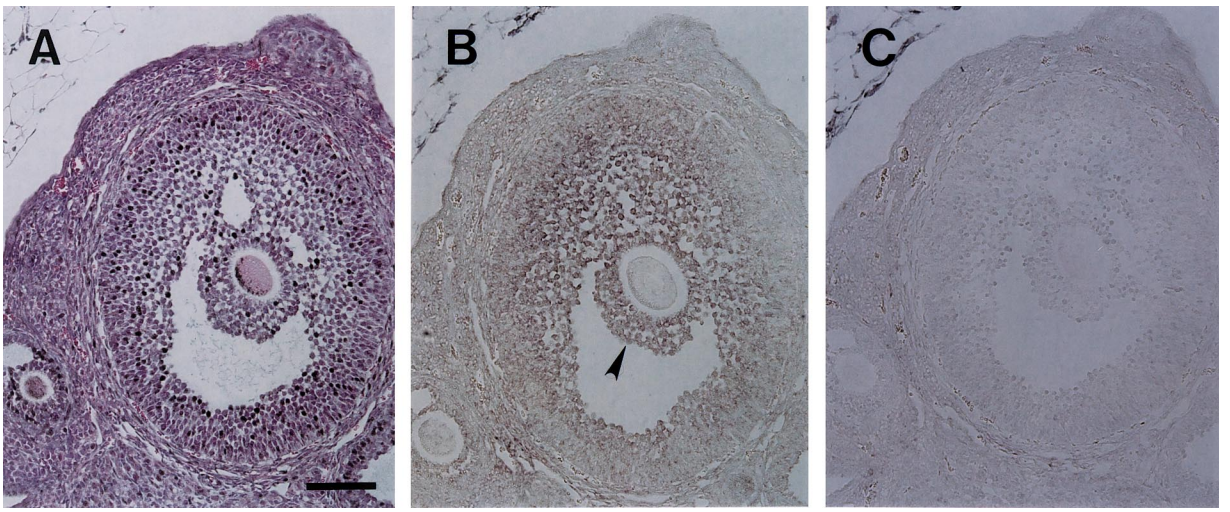

Fig. 1. Bsg mRNA expression in the ovary. In situ hybridization for Bsg mRNA was performed using an ovary from a wild-type mouse. Granulosa cells including cumulus cells (arrow) strongly expressed Bsg mRNA, but mesenchymal cells and the corpora lutea (data not shown) scarcely expressed it. A: Hematoxylin and eosin staining. B: Antisense probe of Bsg. C: Sense probe. Bar, 100 μm.

in a 1% agarose gel, followed by transfer to a nylon membrane. A ^{32}P-labeled probe generated from a cDNA fragment covering the open reading frame of mouse Bsg [1] was hybridized to the membrane. The membrane was washed at room temperature three times in 0.1% SDS, 2×SCC, and then at 55°C twice in 0.1% SDS, 0.1×SCC. Autoradiography was performed with a luminographic analyzer (BAS 2000, Fuji Photofilm).

3. Results

3.1. Female Bsg knock-out mice are infertile, but appear to be normal in ovulation, sex cycle and coital behavior

We examined 16 female mice homozygous for the *Bsg* gene mutation, which had been mated with wild-type male mice, for more than 3 months. Although vaginal plugs were occasionally observed, there was no progeny. The frequency of vaginal plugs in null mutants was comparable to that in wild-type mice (data not shown), indicating that coital behavior was not affected in the null mutants although they exhibited a deficit in spatial memory [13]. The sex cycle, evaluated by means of vaginal smears, was normal (data not shown). Furthermore, the ovaries of Bsg null mutants contained mature follicles and corpora lutea, and superovulation with pregnant mare serum and hCG, which was performed for the null mutants, resulted in successful induction of ovulation [12].

3.2. In vitro and in vivo fertilization

Oocytes obtained by means of superovulation were employed for in vitro fertilization. Null mutant oocytes exhibited a significantly reduced ability of fertilization: successful fertilization was observed in 69/99 (69.7%) of the oocytes from the null mutants and 72/78 (92.3%) of those from the wild-type; $P = 0.0002$ (Table 1). We then examined in vivo fertilization. At 3.5 days p.c. after mating with wild-type male mice, eggs were collected to determine whether or not they had developed into ones with more than two blastomeres. Only one of 39 eggs collected from the null mutants had developed normally, the others remaining unfertilized, many of which appeared degenerated (Table 2). In contrast, 14 of 26 eggs from the wild-type mice had developed normally. The difference was also statistically significant ($P < 0.0001$). The in vivo fertilization results appeared more severely affected than the in vitro ones, probably due to the more rigorous in vivo conditions.

3.3. The genital tract and reach of sperm

The fertilization failure prompted us to examine the intactness of the genital tract. The genital tract, including the vagina, uterus and oviduct, was histologically normal [12]. When a solution of bromophenol blue was injected into the oviduct from the open end at the fimbria, the dye was detected in the uterine lumen (data not shown), indicating that there was no obstruction of the oviduct. This was further confirmed by the fact that oocytes were successfully collected at 2.5 days p.c. by means of oviduct flushing. After mating with wild-type male mice, many sperm were observed in the oviducts of the null mutants: the number and movement of sperm were comparable to those in the oviducts of the wild-type mice (data not shown).

3.4. Bsg expression in the ovary

One possible explanation for the fertilization failure is incomplete oocyte maturation. To examine this possibility, we

Table 1
In vitro fertilization

Zygocity		Numbers of			Rate of fertilization (%)
Oocytes	Sperm	Females	Oocytes	Fertilized eggs	
Null mutant	Wild-type	4	99	69*	69.7
Wild-type	Wild-type	2	78	72*	92.3

*$P = 0.0002$.

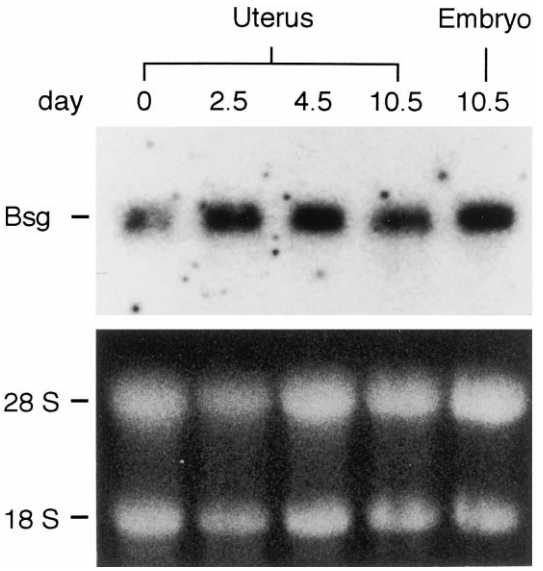

Fig. 2. Northern blot analysis of Bsg expression during early pregnancy. Bsg expression in the uterus was stronger at 2.5 and 4.5 days p.c., and then decreased to 10.5 days p.c. Bsg was strongly expressed in the embryo at 10.5 days p.c. The lower panel shows ethidium bromide staining, which indicates that comparable amounts were loaded on the lanes.

performed in situ hybridization for Bsg mRNA expression in the ovary of wild-type mice. Bsg mRNA was restrictedly expressed in granulosa cells including cumulus cells (Fig. 1), whereas other regions, including the corpora lutea (data not shown) and mesenchyme (Fig. 1), scarcely expressed Bsg mRNA.

3.5. Bsg expression in the uterus

We recently demonstrated that the transfer of normal blastocysts into null mutant uteri resulted in the loss of most embryos during early pregnancy: upon the transfer of a total of 86 wild-type blastocysts into the uteri of six Bsg-null mutant females, only four embryos survived later than the time of implantation, whereas the transfer of 90 blastocysts into wild-type females resulted in 41 embryos being obtained [12]. These data strongly suggest that failure of implantation is one of the prominent causes of female sterility in Bsg null mutants. This led us to further examine Bsg expression in the uterus during early pregnancy.

On Northern blot analysis, Bsg mRNA expression was observed to increase transiently during early pregnancy with peak at around 4.5 days p.c., when implantation takes place (Fig. 2). As we had observed stronger expression of Bsg at the sites of embryo apposition in the endometrium than other sites in coronal sections [12], in situ hybridization for Bsg was further performed on longitudinal sections of uteri around the time of implantation. Bsg expression in the endometrium was again much stronger at sites with embryos in the lumen than at other sites without embryos (data not shown). Furthermore, on fluorescence immunohistochemistry, the Bsg protein was found to be localized in the endometrial epithelium, mostly on the basolateral surface (Fig. 3).

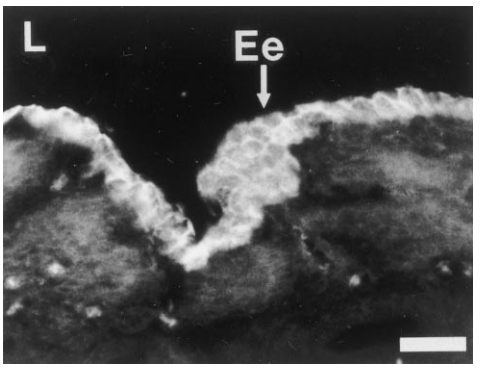

Fig. 3. Bsg protein localization in the uterus. Bsg expression in the uterus at 4.25 days p.c. was observed by immunofluorescence microscopy with anti-Bsg antibodies. Ee, endometrial epithelium. L, uterine lumen. Bar, 25 μm.

4. Discussion

In this paper, we have demonstrated multiple failures in female reproductive processes, that include fertilization and implantation, in Bsg knock-out mice. Multiple female reproductive failures were recently reported for cyclooxygenase 2 knock-out mice, in which ovulation, fertilization, implantation and deciduation are affected [17]. Cyclooxygenase 2 is the rate-limiting enzyme in the biosynthesis of prostaglandins and is associated with the nuclear envelope. Thus, to the best of our knowledge, Bsg is the first reported membrane protein of which a defect leads to multiple female reproductive failures. Since the sex cycle and ovulation are not affected in Bsg null mutants, it is not likely that the failures are due to indirect effects via hormonal failure or defective responsiveness to hormones. Rather than that, Bsg expression in cumulus cells and the endometrial epithelium suggests that Bsg expressed in these regions plays direct roles in oocyte maturation and implantation, respectively.

Since Bsg is expressed in cumulus cells, and interaction between oocyte and cumulus cells is considered important for the production of eggs competent as to fertilization [18], the absence of Bsg in cumulus cells is most likely the cause of fertilization failure in Bsg knock-out mice. In addition, it is of note that cyclooxygenase 2 is expressed in cumulus cells as well as at the site of blastocyst implantation in the uterus [17], this being quite reminiscent of the Bsg expression profile. Cyclooxygenase 2 has also been implicated in tumor invasion [19], and Bsg expressed in tumor cells induces matrix metal-

Table 2
In vivo fertilization

Zygocity		Numbers of			Rate of fertilization (%)
Oocytes	Sperm	Females	Oocytes	Fertilized eggs	
Null mutant	Wild-type	8	39	1*	2.6
Wild-type	Wild-type	4	26	14*	53.8

*$P < 0.0001$.

loproteases in surrounding mesenchymal cells [9]. It remains to be determined whether or not there is any relation between cyclooxygenase 2 and Bsg expression as well as their functional relation.

Many gene targeting studies, including on the genes for cyclooxygenase 2, Hoxa-10, leukemia inhibitory factor (LIF), β1 integrin, EGF receptor, vav, even-skipped, FGF4 and Fug1, have revealed embryonic lethality at the time of implantation or in the early post-implantation period [17,20–31]. However, in most cases, embryonic death was attributed to the loss of the functions of targeted genes in the embryos, but not in the uteri, because expression of the genes was detected in wild-type embryos, and null mutant embryos died in the uteri of heterozygotes. However, in the cases of cyclooxygenase 2, Hoxa-10, and LIF knock-out mice, normal blastocysts cannot become implanted in the uteri of null mutants [17,22–24]. In wild-type mice, LIF is expressed in the uterine endometrial glands specifically on the fourth day of pregnancy [32]. LIF secreted around the time of implantation probably acts on either the blastocysts or the uterus, and thus plays a critical role in implantation. In the cases of Bsg and cyclooxygenase 2, strong expression is detected in the uterine wall at the site of embryo apposition [12,17]. In addition, the expression profile of HB-EGF in the uterus is very reminiscent of those of Bsg and cyclooxygenase 2 [33]. Although elucidation of the biological significance of HB-EGF expression in the uteri awaits a knock-out study on the gene, multiple molecules are now available for investigation of the mechanism of implantation.

It should be noted that the Bsg protein has two important characteristics concerning implantation. First, the extracellular domain of Bsg contains two Ig-like domains. Since many members of the Ig superfamily serve as adhesion molecules, Bsg in the endometrial epithelium could promote the attachment of a blastocyst to the uterus. Second, it has been reported that human Bsg (EMMPRIN) is expressed in human cancer cells, and also induces matrix metalloproteases expression in mesenchymal cells when they encounter cancer cells [9]. Thus, another possible in vivo function of Bsg in implantation is that Bsg in the endometrial epithelium facilitates the production of matrix metalloproteases by trophoblasts, and thereby promotes the invasion of trophoblasts into the uterine wall. The basolateral localization of the Bsg protein in endometrial epithelial cells may indicate the latter possibility is more likely, although the precise mechanism remains to be elucidated.

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References

[1] Miyauchi, T., Kanekura, T., Yamaoka, A., Ozawa, M., Miyazawa, S. and Muramatsu, T. (1990) J. Biochem. 107, 316–323.
[2] Miyauchi, T., Masuzawa, Y. and Muramatsu, T. (1991) J. Biochem. 110, 770–774.
[3] Risau, W., Hallmann, R., Albrecht, U.S. and Henke-Fahle, S. (1986) EMBO J. 5, 3179–3183.
[4] Schlosshauer, B. and Herzog, K.H. (1990) J. Cell Biol. 110, 1261–1274.
[5] Schlosshauer, B. (1991) Development 113, 129–140.
[6] Seulberger, H., Lottspeich, F. and Riau, W. (1990) EMBO J. 9, 2151–2158.
[7] Fadool, J.M. and Linser, P.J. (1993) Dev. Dynamics 196, 252–262.
[8] Fadool, J.M. and Linser, P.J. (1993) J. Neurochem. 60, 1354–1364.
[9] Biswas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T. and Kataoka, H. (1995) Cancer Res. 55, 434–439.
[10] Kasinrerk, W., Fiebiger, E., Stefanova, I., Baumruker, T., Knapp, W. and Stockinger, H. (1992) J. Immunol. 149, 847–854.
[11] Igakura, T., Kadomatsu, K., Taguchi, O., Muramatsu, H., Kaname, T., Miyauchi, T., Yamamura, K., Arimura, K. and Muramatsu, T. (1996) Biochem. Biophys. Res. Commun. 224, 33–36.
[12] Igakura, T., Kadomatsu, K., Kaname, T., Muramatsu, H., Fan, Q.-W., Miyauchi, T., Toyama, Y., Kuno, N., Yuasa, S., Takahashi, M., Senda, T., Taguchi, O., Yamamura, K., Arimura, K. and Muramatsu, T. (1998) Dev. Biol. (in press).
[13] Naruhashi, K., Kadomatsu, K., Igakura, T., Fan, Q.-W., Kuno, N., Muramatsu, H., Miyauchi, T., Hasegawa, T., Itoh, A., Muramatsu, T. and Nabeshima, T. (1997) Biochem. Biophys. Res. Commun. 236, 733–737.
[14] Fan, Q.-W., Yuasa, S., Kuno, N., Senda, T., Kobayashi, M., Muramatsu, T. and Kadomatsu, K. (1998) Neurosci. Res. (in press).
[15] Kadomatsu, K., Anzano, M.A., Slayter, M.V., Winokur, T.S., Smith, J.M. and Sporn, M.B. (1993) Cancer Res. 53, 1480–1483.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[17] Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M. and Dey, S.K. (1997) Cell 91, 197–208.
[18] Epigg, J.J. (1991) BioEssays 13, 569–574.
[19] Tsujii, M., Kawano, S. and Dubois, R.N. (1997) Proc. Natl. Acad. Sci. USA 94, 3336–3340.
[20] Dinchuk, J.E., Car, B.D., Focht, R.J., Johnston, J.J., Jaffee, B.D., Covington, M.B., Contel, N.R., Eng, V.M., Collins, R.J., Czerniak, P.M., Gorry, S.A. and Trzaskos, J.M. (1995) Nature 378, 406–409.
[21] Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A. and Smithies, O. (1995) Cell 83, 473–482.
[22] Satokata, I., Benson, G. and Maas, R. (1995) Nature 374, 460–463.
[23] Benson, G.V., Lim, H., Paria, B.C., Satokata, I., Dey, S.K. and Maas, R.L. (1996) Development 122, 2687–2696.
[24] Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Köntgen, F. and Abbondanzo, S.J. (1992) Nature 359, 76–79.
[25] Fässler, R. and Meyer, M. (1995) Genes Dev. 9, 1896–1908.
[26] Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K. and Harris, R.C. (1995) Science 269, 230–234.
[27] Zmuizdzinas, A., Fischer, K.D., Lira, S.A., Forrester, L., Bryant, S., Bernstein, A. and Barbacid, M. (1995) EMBO J. 14, 1–11.
[28] Spyropoulos, D.D. and Capecchi, M.R. (1994) Genes Dev. 8, 1949–1961.
[29] Feldman, B., Poueymirou, W., Papaioannou, V.E., DeChiara, T.M. and Goldfarb, M. (1995) Science 267, 246–249.
[30] DeGregori, J., Russ, A., von Melchner, H., Rayburn, H., Priyaranjan, P., Jenkins, N.A., Copeland, N.G. and Ruley, H.E. (1994) Genes Dev. 8, 265–276.
[31] Cross, J.C., Werb, Z. and Fisher, S.J. (1994) Science 266, 1508–1518.
[32] Bhatt, H., Brunet, L.J. and Stewart, C.L. (1991) Proc. Natl. Acad. Sci. USA 88, 11408–11412.
[33] Das, S.K., Wang, X.N., Paria, B.C., Damm, D., Abraham, J.A., Klagsbrun, M., Andrews, G.K. and Dey, S.K. (1994) Development 120, 1071–1083.