Expression of basigin in reproductive tissues of estrogen receptor- α or - β null mice

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Abstract

Basigin plays important roles in both male and female reproduction because basigin (*Bsg*) null male and female mice are infertile. The aim of the present study was to determine whether basigin expression in reproductive organs requires estrogen receptor- α (ESR1, ER α) or - β (ESR2, ER β). Expression of basigin protein in the testis, ovary, and male and female reproductive tracts was studied in adult wild-type (WT), *Esr1*-null (α ERKO), and *Esr2*-null (β ERKO) mice by immunohistochemistry and immunoblotting. Basigin mRNA levels in ovary and uterus were examined by quantitative RT-PCR. In females, basigin protein expression was observed mainly in granulosa and interstitial cells of the ovary and epithelial cells of the proximal oviduct in all genotypes. Basigin protein was also expressed in the uterine epithelium at proestrus and estrus in WT and β ERKO mice but not in α ERKO mice. However, a higher level of basigin mRNA was observed in uteri of α ERKO mice compared with WT and β ERKO mice. In males, basigin was expressed in Leydig cells and all germ cells except spermatogonia in all genotypes. Basigin was present in epithelial cells lining the efferent ductules in WT and β ERKO mice, but expression was greatly reduced in α ERKO mice. In epididymal ducts, basigin expression was observed in epithelial cells in the caput and cauda in all genotypes. These data suggest that expression of basigin protein requires ESR1, but not ESR2, in the uterus and efferent ductules, but is independent of estrogen receptor in the ovary, oviduct, testis, and epididymis.

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Introduction

Basigin, also known as EMMPRIN, CD147, OX-47, and CE9, is a highly glycosylated transmembrane protein with two immunoglobulin-like domains (Muramatsu & Miyachi 2003). Basigin is expressed in a broad range of tissues and involved in a number of physiological and pathological processes such as spermatogenesis (Igakura *et al.* 1998, Toyama *et al.* 1999), fertilization (Kuno *et al.* 1998), embryo implantation (Igakura *et al.* 1998), tumor invasion (Biswas *et al.* 1995, Zucker *et al.* 2001, Tang *et al.* 2005), immune responses (Pushkarsky *et al.* 2001), inflammation (Igakura *et al.* 1996, Kontinen *et al.* 2000, Yurchenko *et al.* 2002, Arora *et al.* 2005), and neural functions such as vision, behavior, memory, and olfaction (Naruhashi *et al.* 1997, Fan *et al.* 1998, Hori *et al.* 2000, Ochrietor *et al.* 2001, Philp *et al.* 2003). It is well established that basigin overexpressed on the surface of tumor cells plays significant roles in tumor growth, angiogenesis, invasion, and metastasis by stimulating expression of matrix metalloproteinases

(Biswas 1984, Kataoka *et al.* 1993, Guo *et al.* 1997, Tang *et al.* 2005), vascular endothelial growth factor (Tang *et al.* 2005), and hyaluronan (Marieb *et al.* 2004). Basigin is considered a marker of poor outcome of ovarian carcinoma (Davidson *et al.* 2003).

Basigin is abundant in the reproductive organs, including the testis, ovary, and uterus (Igakura *et al.* 1998, Kuno *et al.* 1998, Chen *et al.* 2001, Noguchi *et al.* 2003, Chang *et al.* 2004), and plays essential roles in both male and female reproduction. Targeted disruption of the *Bsg* gene in mice results in the failure of spermatogenesis and defects in fertilization and implantation (Igakura *et al.* 1998, Kuno *et al.* 1998). The steroid hormone, estradiol, acting via the nuclear receptors, estrogen receptor- α (ESR1, ER α) and - β (ESR2, ER β), is fundamental to the regulation of numerous genes in reproductive tissues. Basigin expression in the mouse uterine epithelium is reportedly upregulated by estrogen (Xiao *et al.* 2002), but the role of ESR1 and ESR2 in the regulation of basigin expression remains unknown.

Figure 1 Basigin immunoreactivity in the uteri (estrus) of WT (A) and *Bsg*-null (B) female mice. Uterine luminal (A, big arrow) and glandular (A, big arrow head) epithelia and erythrocytes (A, small arrow head) in the endometrium are strongly positive for basigin in the WT mouse at estrus. Basigin is also positive in myometrium (A, small arrow), whereas there is no basigin immunoreactivity in the *Bsg*-null uterus (B). Scale bar=50 μ m.

The goal of the present study was to compare the expression of basigin in the reproductive tissues of wild-type (WT), *Esr1*-null (α ERKO), and *Esr2*-null (β ERKO) mice to determine the contribution of each estrogen receptor isoform to basigin expression. The results showed that ESR1 not ESR2 was required for the proper expression of basigin protein in the uterus and efferent ductules, while basigin protein expression in the ovary, oviduct, testis, and epididymis was estrogen receptor-independent.

Results

Antibody specificity

Basigin immunoreactivity was identified on the apical and bilateral surface of uterine epithelial cells in endometrium of WT mice (Fig. 1A), consistent with a previous report (Xiao *et al.* 2002). We also observed basigin immunoreactivity on the surface of erythrocytes in the blood vessels and myometrium in the uteri (Fig. 1A). Furthermore, no basigin immunoreactivity was observed among uterine sections from the *Bsg*-null mice (Fig. 1B), confirming the specificity of the antibody for mouse basigin.

Basigin protein expression in the reproductive tissues of WT, α ERKO, and β ERKO females

Basigin immunoreactivity in WT ovaries was intense on the surface of granulosa cells and interstitial cells but weak or absent in theca cells and corpus luteum cells (Fig. 2A–C and Table 1). In the corpora lutea of WT ovaries, strong basigin immunoreactivity was identified on the surface of erythrocytes in the blood vessels, not in corpus luteum cells (Fig. 2C). Basigin immunoreactivity was also detected on the surface of cumulus oophorus cells surrounding the ovulated oocytes that were retained in the oviduct of WT mice at estrus (Fig. 3A). Similarly, strong immunoreactivity for basigin was observed in granulosa and interstitial cells in α ERKO and β ERKO ovaries (Fig. 2D and E; Table 1). As expected, ovaries from α ERKO mice exhibited large, hemorrhagic, and cystic follicles as well as lacked corpora lutea (Couse & Korach 1999), but no aberrant basigin expression was associated with these pathologies.

Strong basigin immunoreactivity was observed on the basolateral surface of ciliated and non-ciliated epithelial cells of the proximal oviducts from WT mice (Fig. 3A and Table 1). This expression pattern was not

Figure 2 Basigin immunoreactivity in ovaries of WT (A–C), α ERKO (D), and β ERKO (E) mice. Ovarian sections were stained with goat anti-mouse basigin antibody (A–E) or goat non-specific IgG (F). In the ovaries of WT mice, basigin is strongly positive in the granulosa cells of follicles and interstitial cells. In the corpora lutea, basigin is expressed by erythrocytes (arrows in C) in the blood vessels, not by the corpus luteum cells (C). A similar expression pattern is observed in α ERKO (D) and β ERKO (E) ovaries. Scale bar=250 μ m for A and F, and 25 μ m for B–E. Stars in A represent corpus luteum; arrows in C represent erythrocytes.

Table 1 Summary of basigin expression in ovary, testis, and reproductive tracts of wild-type (WT), α ERKO, and β ERKO mice. Three or more mice of each different genotype were used for immunohistochemistry analysis. Three slides per tissue and at least five fields within a slide were evaluated by two observers.

Organ	Cells	WT	α ERKO	β ERKO
Ovary	Granulosa cells	++	++	++
	Interstitial cells	++	++	++
	Theca cells	$\pm$	$\pm$	$\pm$
	Corpus luteum	—	NA	—
Oviduct	Epithelial cells	++	++	++
Uterus	Epithelial cells	++ (proest, est)	—	++ (proest, est)
	Stroma cells	—	—	—
	Sertoli cells	$\pm$	$\pm$	$\pm$
Testis	Leydig cells	++	++	++
	Spermatogonia	—	—	—
	Spermatocytes	++	++	++
	Spermatid	++	++	++
	Sperm	++	++	++
	Ciliated epithelial cells	++	$\pm$	++
	Non-ciliated epithelial cells	++	$\pm$	++
Efferent ductule	Initial segment	$\pm$	$\pm$	$\pm$
	Caput	+	+	+
Epididymis	Corpus	$\pm$	$\pm$	$\pm$
	Cauda	+	+	+

++, strongly positive; +, positive; $\pm$, weakly positive; —, negative; proest, proestrus; est, estrus; NA, not applicable.

affected by the stage of estrous cycle (data not shown) or the loss of either estrogen receptor isoform (Fig. 3B and C; Table 1). On the other hand, basigin immunoreactivity clearly fluctuated throughout the estrous cycle in the uteri of WT females. Basigin immunoreactivity was high on the apical and lateral surfaces of the luminal and glandular epithelia at proestrus and estrus (Fig. 4A and B; Table 1), while that in endometrium was weak or absent at metestrus (Fig. 4C) and diestrus (Fig. 4D). Fibroblasts in the

uterine stroma were consistently negative for basigin, while basigin immunoreactivity was seen on the surface of erythrocytes and myometrium in the uteri at all stages of estrous cycle (Fig. 4A–D). The expression pattern of basigin in uteri of β ERKO mice was comparable to that in WT mice (Fig. 4F and Table 1). In the α ERKO uteri, basigin was undetectable in the luminal and glandular epithelia (Fig. 4E, G and H; Table 1), although it was still detectable in the erythrocytes and myometrium (Fig. 4E, G and H).

Figure 3 Basigin immunoreactivity in oviducts of WT (A), α ERKO (B), and β ERKO (C) mice. Sections were stained with goat anti-mouse basigin antibody (A–C) or goat non-specific IgG (D). Basigin expression is evident on the surface of ciliated and non-ciliated epithelial cells of WT, α ERKO, and β ERKO mice. Basigin expression is observed on the surface of cumulus oophorus cells (A, arrow) surrounding the ovulated oocyte (A, arrow head) that were retained in the oviduct of WT mice at estrus. Scale bar=50 μ m for A–C and 100 μ m for D.

Figure 4 Basigin immunoreactivity in uteri of WT (A–D), α ERKO (E, G, and H), and β ERKO (F) mice. Uteri were collected at proestrus (A), estrus (B), metestrus (C), or diestrus (D) from WT mice, and at estrus from β ERKO mice (F). Sections were stained with goat anti-mouse basigin antibody (A–H) or goat non-specific IgG (I). Basigin immunoreactivity is intense in luminal and glandular epithelia of WT mice at proestrus (A), estrus (B), weak at metestrus (C), and very low or absent at diestrus (D). Basigin expression is seen on the surface of erythrocytes (arrows in A–D) and myometrium (data not shown) in the WT uteri at all stages of estrous cycle. In the α ERKO uteri, basigin is not observed in the luminal or glandular epithelium, whereas basigin immunoreactivity is detectable in erythrocytes (arrows in G) and myometrium (arrow heads in H). Scale bar=50 μ m for A–D, F, and I, 25 μ m for E, and 15 μ m for G and H.

Basigin protein expression in the reproductive tissues of WT, α ERKO, and β ERKO males

Basigin immunoreactivity in WT testes was seen on the cell surface of Leydig cells, spermatocytes, spermatids, and sperm tails, while undetectable in spermatogonia (Fig. 5A and A'; Table 1). Weak immunoreactivity was detected on the surface of Sertoli cells (Fig. 5A and A'; Table 1). Basigin immunoreactivity in α ERKO and β ERKO testes was comparable to that in WT males (Fig. 5B and C; Table 1), despite the flattened epithelia and diluted lumen that are characteristic abnormalities of the testes of α ERKO mice.

The efferent ducts of WT and β ERKO mice exhibited strong basigin immunoreactivity on the basolateral surface of ciliated and non-ciliated epithelial cells (Fig. 6A and C; Table 1), while this immunoreactivity was reduced or absent in the efferent ductules of α ERKO males, all of which were dilated and lined with cuboidal epithelia (Fig. 6B and Table 1).

Basigin expression varied along the epididymal duct in WT mice. Moderate basigin immunoreactivity was localized on the lateral surface of epithelial cells in the caput (Fig. 7B) and apical surface in the cauda (Fig. 7D), while weak immunoreactivity was seen on lateral surfaces of epithelial cells in the initial segment (Fig. 7A) and apical surface in the corpus (Fig. 7C). Basigin expression in the α ERKO and β ERKO epididymis was comparable to that in WT males (Table 1).

Basigin protein and mRNA levels in uteri of WT, α ERKO, and β ERKO mice

Our immunostaining clearly shows the absence of basigin protein expression in the uterine luminal and glandular epithelia of α ERKO mice (Fig. 4E and G). We further quantified basigin protein levels in α ERKO uteri by immunoblotting. Basigin protein levels of α ERKO uteri were greatly reduced to around 40% of those of WT uteri (Fig. 8A). These data underestimate the down-regulation of basigin protein in uterine epithelium of α ERKO mice, because basigin expression by erythrocytes and myometrium of α ERKO uteri is comparable to that of WT uteri (Fig. 4E, G, and H).

The estrogen–ESR1 complex directly regulates the transcription of some genes including lactoferrin and progesterone receptor. Although our immunohistochemistry and immunoblotting results suggest that ESR1 is required for proper basigin protein expression in uteri, especially in uterine epithelium, it is not clear whether the signaling mediated by ESR1 regulates basigin mRNA levels in uteri. To answer this question, we examined basigin mRNA levels in uteri of α ERKO and β ERKO mice. Since lactoferrin transcript is regulated by the action of ESR1, we used lactoferrin as a control gene. As expected, lactoferrin mRNA levels in the uteri of α ERKO mice were only 20% of those of WT mice (Fig. 8B). In contrast to the absence of basigin protein expression in uterine epithelium of α ERKO mice, basigin mRNA levels in uteri of α ERKO mice were threefold increased

Figure 5 Basigin immunoreactivity in testes of WT (A and A'), α ERKO (B and B'), and β ERKO (C and C') mice. Sections were stained with goat anti-mouse basigin antibody (A–C) or goat non-specific IgG (D). A', B', and C' are the enlarged views of the highlighted areas in A–C respectively. In WT mice, strong basigin immunoreactivity is observed on the cell surface of Leydig cells, spermatocytes, spermatids, and sperm tails, while undetectable in spermatogonia. The same pattern of basigin expression is observed in α ERKO (B) and β ERKO (C) mice. Big arrow head, Sertoli cells; big arrow, spermatocytes; small arrow, round spermatids; small arrow head, spermatogonia. Scale bar=50 μ m for A–D, and 15 μ m for A', B', and C'.

compared with those of WT mice (Fig. 8B). As expected, basigin mRNA levels in uteri of β ERKO mice and ovaries of α ERKO and β ERKO mice were comparable to those of WT mice (Fig. 8B).

Discussion

Basigin is a glycosylated transmembrane protein that is expressed in multiple reproductive organs and plays critical roles in male and female fertility, as evidenced by the infertility in *Bsg*-null mice (Igakura *et al.* 1998, Kuno *et al.* 1998). The present study was conducted to determine whether basigin expression in male and female reproductive organs requires the actions of ESR1 or ESR2. By comparing the level and pattern of basigin expression in the reproductive tissues of WT, α ERKO, and β ERKO mice, we identified the uterine epithelium in females and the efferent ductule epithelium in males as tissues in which basigin expression requires functional ESR1, while expression in the gonads, oviduct, and epididymis is estrogen receptor-independent.

Our finding of markedly high basigin levels in ovarian granulosa cells, interstitial cells, and ovarian surface epithelial cells but the absence of expression in luteal cells is consistent with the report of Kuno *et al.* (1998). In contrast, basigin mRNA and protein were detectable in both new corpora lutea and corpora lutea from previous cycles in the rat ovary (Smedts & Curry 2005), suggesting that basigin expression in corpus lutea is species-specific. Still, the ovaries of *Bsg*-null mutant mice exhibit normal folliculogenesis and corpus lutea (Kuno *et al.* 1998), as well as normal progesterone levels on day 8 of

pseudopregnancy (L. Chen, unpublished observations). However, the reduced rate of fertilization *in vivo* among *Bsg*-null oocytes (Kuno *et al.* 1998) suggests a function of basigin in granulosa cells that is critical to oocyte maturation and function. Our demonstration that basigin expression in mouse ovaries was not altered by the absence of either ESR1 or ESR2 indicates that estrogen signaling is not required for basigin expression in the ovary. Instead, basigin expression in granulosa cells may depend on epidermal growth factor or amphiregulin (Yoshino *et al.* 2006), both of which are produced by granulosa cells and have been shown to induce basigin expression in NS2T2A1 human breast tumor cells (Menashi *et al.* 2003).

Basigin is reportedly overexpressed in all malignant ovarian tumors including serous adenocarcinoma, mucinous adenocarcinoma, yolk sac tumor, clear cell carcinoma, and granulosa cell tumors (Jin *et al.* 2006). The level of basigin expressed by ovarian cancer cells is positively related with cancer cell invasive ability (Millimaggi *et al.* 2007). Several ovarian epithelial tumor cell lines have been demonstrated to release basigin protein by microvesicle shedding. Microvesicles shed from ovarian carcinoma cells stimulate proangiogenic activities of human umbilical vein endothelial cells in a basigin-dependent manner, indicating the significant role of basigin in ovarian tumor invasion and metastasis (Millimaggi *et al.* 2007).

Basigin expression in the uterine endometrium was localized to the luminal and glandular epithelia and clearly fluctuated during the cycle such that the expression was markedly high at proestrus and estrus.

Figure 6 Basigin immunoreactivity in efferent ductules of WT (A), α ERKO (B), and β ERKO (C) mice. Sections were stained with goat anti-mouse basigin antibody (A–C) or goat non-specific IgG (D). Intense basigin immunoreactivity is observed in the ciliated and non-ciliated epithelial cells of WT (A) and β ERKO (C) mice. Basigin immunoreactivity is greatly diminished or absent in epithelial cells of α ERKO efferent ductules (B, arrow). Scale bar = 50 μ m for A–C and 200 μ m for D.

Further evidence of estrogen regulation of basigin protein expression in the uterine epithelium, and the importance of ESR1 in mediating this effect, was the total lack of basigin protein in the uterine epithelium of *Esr1*-null females. Paradoxically, we observed a significant increase, instead of a decrease, in basigin mRNA levels in the uteri of *Esr1*-null females. These apparently contradictory observations suggest that the signaling mediated by ESR1 must be necessary for basigin protein translation, instead of mRNA transcription. Knockout of *Esr1* results in a lower basigin protein expression in the uteri, which may lead to a compensatory response with increases in basigin mRNA levels.

MicroRNAs (miRNAs), a group of ~22 nucleotide-long non-coding RNAs, powerfully negatively regulate gene expression by targeting mRNAs at the post-transcriptional level. miRNAs inhibit the translation of or degrade their target mRNAs by binding to the 3'-UTR of target mRNAs (Ambros 2004). Each miRNA targets ~200 transcripts directly or indirectly (Zhang *et al.* 2006), and ~30% of human genes are potential targets of miRNA (Yu *et al.* 2006). Interestingly, some miRNAs are regulated by steroid hormones. For example, estrogen downregulates miR-21 expression, which leads to increased expression of miR-21-target genes at the protein levels (Wickramasinghe *et al.* 2009). It is possible that some miRNAs that are downregulated by estrogen suppress basigin mRNA translation in uteri. Blocking estrogen signaling by knocking out *Esr1* could cause an increase in some miRNAs, which in turn inhibits translation of basigin mRNA.

In contrast, and despite the substantial level of ESR1 expression in the oviduct epithelium, basigin expression

in the oviduct was not affected by the loss of functional ESR1 nor did it overtly fluctuate during the estrous cycle in WT females. These data illustrate very nicely the differential regulation of basigin expression between epithelial cell types of the uterus and oviduct in mice.

In males, the intense basigin expression observed in Leydig cells and all stages of germ cells except spermatogonia is in agreement with previous reports (Igakura *et al.* 1998, Maekawa *et al.* 1998). Not surprisingly, *Bsg*-null mice exhibit a complete arrest of spermatogenesis at the metaphase of the first meiosis (Igakura *et al.* 1998). ESR1 and ESR2 are expressed in various somatic and germ cell types of the testis (Zhou *et al.* 2002). However, deletion of either estrogen receptor isoform does not affect basigin expression in the testis, as expression was not altered in α ERKO or β ERKO mice.

The epithelial cells of the efferent ductules are reported to have a markedly high concentration of ESR1 (Schleicher *et al.* 1984, Cooke *et al.* 1991, Zhou *et al.* 2002). Furthermore, there is convincing evidence that ESR1-mediated estrogen actions are required to maintain the fluid resorption of the efferent ducts (Hess *et al.* 1997). Herein, we found that basigin expression is localized to the basolateral surface of efferent ductule epithelial cells, similar to the reported site of the Na^+ , K^+ -ATPase that is thought to be important in fluid absorption (Lee *et al.* 2001). Still, *Bsg*-null mice possess efferent ducts that exhibit normal morphology and no overt functional deficits (Nakai *et al.* 2006). *Bsg*-null mice do, however, exhibit impaired expression of monocarboxylate transporter 1 (MCT1; Nakai *et al.* 2006), a transmembrane protein responsible for the transport of monocarboxylates across the plasma

Figure 7 Basigin immunoreactivity in epididymis of WT mice. Moderate basigin immunoreactivity is observed on the lateral surface of epithelial cells in caput (B) and apical surface in cauda (D), while weak immunoreactivity is detected on the lateral surface of epithelial cells in the initial segment (A) and apical surface in the corpus (C). Scale bar = 50 μ m.

membrane. Deletion of *Esr1* may lead to impaired MCT1 expression as well as Na^+/K^+ -ATPase, which may in turn contribute to the invariable phenotype of poor fluid resorption in *Esr1*-null males.

In summary, we have employed estrogen receptor-null mice to demonstrate that basigin expression is estrogen- and ESR1-dependent in some reproductive tissues (e.g. uterine and efferent ductile epithelia), but not in all reproductive tissues (e.g. gonads, oviduct, and epididymis) in both sexes. Future studies are required to identify the factors that regulate basigin expression in those reproductive tissues in which estrogen receptor-mediated estrogen actions appear to have little influence.

Materials and Methods

Animals

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were pre-approved by the NIEHS IACUC. The generation of mice null for the *Esr1* (ER α) or *Esr2* (ER β) genes was in a C57BL6/J background and has been described previously (Lubahn *et al.* 1993, Kregge *et al.* 1998). Estrous cycle phase for WT and *Esr2*-null females was determined by vaginal smear over a minimum period of 1 week. Three or more mice for each genotype at different estrous cycles were used for this study.

Tissue collection

All mice were killed by carbon dioxide asphyxiation. For immunohistochemistry uteri, ovaries and oviducts were fixed in 10% formalin, whereas testes and efferent ductules were fixed in Bouin's solution overnight. All tissues were then

processed for paraffin embedding. For immunoblotting and quantitative RT-PCR analyses, uteri and ovaries were snap-frozen with nitrogen and kept at -80°C for future use.

Immunohistochemistry

Tissues were sectioned at 5 μ m and mounted on poly-L-lysine-coated slides. Sections were boiled in 10 mM citrate buffer (pH 6.0) for 10 min to promote antigen retrieval. Endogenous peroxidase activity was blocked in methanol containing 0.3% hydrogen peroxide for 15 min. After blocking with 5% normal rabbit serum in PBS for 20 min, sections were incubated with 2 μ g/ml goat polyclonal antibody against mouse basigin (R&D, Minneapolis, MN, USA) at 4 $^\circ\text{C}$ overnight. Normal goat IgG (2 μ g/ml) was applied as a negative control. After washing in PBS, sections were incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) diluted at 1:100 with PBS for 1 h at room temperature. Sections were then incubated in ABC solution (Vector Labs) for 45 min, reacted with metal-3,3'-diaminobenzidine (Sigma) for 3 min, and counterstained with hematoxylin.

Immunoblotting

Frozen whole uteri isolated from WT and α ERKO 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 (Pierce, Rockford, IL, USA). Twenty micrograms of total proteins were resolved by 10% SDS-PAGE. The gels were transferred to nitrocellulose membranes, blocked in 5% non-fat dry milk, and probed with 0.2 μ g/ml goat polyclonal antibody against mouse basigin (R&D) for overnight at 4 $^\circ\text{C}$. The membranes were washed and incubated with the HRP-conjugated donkey anti-goat IgG

Figure 8 Basigin protein and mRNA levels in uteri of WT and ERKO mice. Uterine protein or RNA was isolated from whole uteri and subjected to immunoblotting (A) or quantitative RT-PCR analyses (B). For immunoblotting, basigin bands were normalized to GAPDH bands. Basigin protein levels of αERKO uteri are 40% of those of WT mice, while basigin mRNA levels of αERKO uteri were threefold increased compared with those of WT (B). Basigin mRNA levels in uteri and ovaries of βERKO mice were comparable to those of WT mice (B). The data are presented as fold changes relative to tissues of WT mice, and the numbers of samples are indicated. Data are means $\pm$ S.E.M. The asterisk indicates a statistical difference ($P < 0.05$) compared with WT tissues.

antibody (Santa Cruz Company, Santa Cruz, CA, USA) at 1:3000 dilution for 45 min at room temperature. The bound secondary antibody was detected using chemiluminescent reagents (Pierce). The same membranes were stripped and reprobed with anti-GAPDH antibody (Cell Signaling, Boston, MA, USA) 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 basigin bands were normalized for loading differences using measurements of GAPDH. Graphs represent means and S.E.M.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from mouse uteri and ovaries using TRIzol according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed using the RETROscript First Strand cDNA Synthesis Kit, and quantitative RT-PCR analysis was performed using TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Atlanta, GA, USA). Primer and

probe set for basigin were designed using Primer Express software (Perkin-Elmer, Waltham, MA, USA). For basigin cDNA amplification, 200 nM forward primer (5'-TGGA-CCGTGTTACATCCAT-3'), 200 nM reverse primer (5'-CCC-ATCAACAGAGAGCGAACT-3'), and 200 nM probe (5'-CCGCCTACCGTCAGCATGCAGC-3') were used. Lactoferrin was amplified using the 20 $\times$ Assays-on-Demand Gene Expression Assays purchased from Applied Biosystems. Relative mRNA levels were calculated using the relative standard curve method. Standard curves were generated from dilution series constructed from a 'reference sample', which was cDNA from a WT mouse uterus or ovary. Quantitative PCR was performed on both experimental samples and reference standards. Relative values for basigin, lactoferrin, and ribosomal 18S abundance in all samples were extrapolated from the standard curves from the reference standards. Relative mRNA levels were normalized to 18S. To show changes in gene expression relative to control levels (WT mouse tissue, set as 1), each of the samples was normalized to the average of the control groups.

Statistical analysis

Results are presented as the mean $\pm$ S.E.M. of at least three samples. Statistical analyses were performed by ANOVA. Values of $P < 0.05$ were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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