Ablation of the Sam68 gene impairs female fertility and gonadotropin-dependent follicle development

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Received June 22, 2010; Revised and Accepted September 21, 2010

Sam68 is a multifunctional RNA-binding protein highly expressed in the gonads, whose ablation causes male infertility. Herein, we have investigated Sam68 expression in the adult ovary and its function in female fertility. Immunohistochemistry showed that Sam68 was localized in the nucleus of oocytes and follicular cells at all stages of folliculogenesis. Sam68−/− females were severely subfertile, and they showed a delay in the age of first pregnancy, increased breeding time for successful pregnancy and yielded smaller litters. Morphological analyses indicated a significant reduction in the number of secondary and pre-antral follicles in the ovary. These defects were associated with alteration of oestrous cycles and a reduced number of ovulated oocytes, which were only partially restored by the administration of exogenous gonadotropins. Crosslinking/immunoprecipitation experiments showed that Sam68 directly binds the mRNAs for the follicle-stimulating hormone (FSH) and the luteinizing hormone receptors (Fshr and Lhcgr), which were down-regulated in ovaries of adult knockout females. Stimulation of immature females with FSH-like pregnant mare serum gonadotropin (PMSG), or of follicular cells with the FSH second messenger analogue 8Br-cAMP, caused the upregulation of Sam68. The increase in Sam68 levels paralleled that of the Fshr and Lhcgr mRNAs in the pre-ovulatory follicle and was required to allow accumulation of these transcripts in follicular cells. These studies identify a new crucial function for Sam68 in the regulation of female fertility and indicate that this protein is required to insure proper expression of the gonadotropin receptor transcripts in pre-ovulatory follicles in adult ovary.

INTRODUCTION

Sam68 belongs to the evolutionarily conserved signal transduction activator of RNA (STAR) family of RNA-binding proteins (RBPs) (1), which play key roles during cell differentiation and development (2,3). STAR proteins are characterized by a GSG (GRP33/Sam68/GLD-1) RNA-binding domain that is also required for protein homodimerization (1–3). Moreover, regulatory regions outside the GSG domain allow protein–protein interactions and contain sites for post-translational modifications of STAR proteins (1). In particular, Sam68 interacts with several proteins containing Src homology 3 (SH3) and SH2 domains through proline-rich sequences and tyrosine-phosphorylated residues, respectively. Sam68 was originally described as a scaffold protein recruited in various signal transduction pathways (1,3). However, Sam68 also modulates several steps of RNA metabolism such as alternative splicing (4–8), nuclear export and cytoplasmic utilization or translation of viral and cellular mRNAs (1,3).

The generation of a mouse knockout (KO) model has recently unveiled the physiological functions of Sam68. Although 20–30% of the Sam68−/− mice die perinatally, most KO animals survive through adulthood, indicating that this RBP is not strictly required for viability (9). Sam68−/− mice are characterized by an altered bone metabolism, as they do not display loss of bone mass upon ageing. This phenotype is probably due to a role for this RBP in bone marrow mesenchymal cell differentiation, because Sam68 expression promoted adipocyte differentiation, whereas its deletion	

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favored the osteogenic pathway (9). Sam68<sup>−/−</sup> mice also displayed mild motor coordination defects (10), suggesting a neuronal function for this STAR protein. Furthermore, Sam68 haploinsufficiency was shown to delay mammary tumorigenesis in vivo (11), in line with the growing body of information about the role of Sam68 in human cancers, which involves its ability to modulate alternative splicing (6,8), transcription (12) and protein–protein interactions (13). Thus, many roles played by Sam68 in the cell allow this versatile protein to affect different biological processes, and modulation of its activity and/or expression levels probably affects various pathological situations.

Sam68 is abundantly expressed in the testis (14), and Sam68<sup>−/−</sup> male mice are infertile (15). Spermatogenesis is seriously compromised in Sam68<sup>−/−</sup> males, and it was shown that this RBP promotes translation of a subset of mRNAs required for the production of fertile gametes (15). In contrast, although Sam68 is highly expressed in mouse ovary (3), much less is known about its role in female fertility. Ovarian development requires the coordinated differentiation of germ cells and somatic cells in functional units termed follicles (16). In the mouse, primordial germ cells/oogonia enter the prophase of the first meiotic division and differentiate into primary oocytes in the foetal ovary. Around birth, oocytes are surrounded by pre-granulosa cells and become individually enclosed in primordial follicles, which represent the reservoir for all growing follicles during the lifespan of the animal (17). Primordial follicles are recruited to grow into primary and secondary follicles, when they begin to express the gonadotropin and steroid receptors (18,19). At puberty, all the subsequent steps of follicle development are cyclically regulated by the pituitary gonadotropins (20–22). Follicle-stimulating hormone (FSH) binds to a specific receptor (FSH-R) expressed by follicular cells and promotes cell proliferation and differentiation into antral pre-ovulatory follicles (19). Next, binding of luteinizing hormone (LH) to its receptor (LHR) on the differentiated follicular cells induces a cascade of intracellular signalling events, leading to changes in gene expression that promote ovulation (19,23).

Herein, by investigating the reproductive phenotype of female Sam68<sup>−/−</sup> mice, we have discovered a novel, unexpected role for Sam68 in female reproduction. Our results suggest the involvement of Sam68 in the pathway triggered by gonadotropins and indicate that expression of this protein in ovarian follicles is required to insure their proper hormonal response and ovulation.

RESULTS

Expression of Sam68 in developing ovarian follicles

Sam68 is abundantly expressed in the whole ovary and in fully grown oocytes (3,24). In order to clarify the role of Sam68 in female fertility, we first investigated its expression at different stages of folliculogenesis in the adult ovary. We observed that Sam68 is expressed at high levels in all types of follicles compared with ovarian interstitial cells (Fig. 1A and Supplementary Material, Fig. S1). Specifically, Sam68 localizes in the nucleus of both oocytes and follicular cells of primordial (Supplementary Material, Fig. S1Ca), primary (Supplementary Material, Fig. S1Cb), secondary (Supplementary Material, Fig. S1Cc) and early antral (Supplementary Material, Fig. S1Cd) follicles, without appreciable changes in its expression levels. The specificity of the antibody was assessed by comparing the signal obtained with follicles from Sam68<sup>−/−</sup> ovary (Supplementary Material, Fig. S1Ca–c’).

Ablation of Sam68 impairs female fertility

Sam68<sup>−/−</sup> male mice are sterile due to impairment of spermatogenesis (15). In order to investigate whether ablation of Sam68 also affects female fertility, we crossed wild-type (WT) and KO females with Sam68<sup>−/−</sup> males of proven fertility. Females were housed with males starting at 4 weeks of age and monitored continuously for the presence of the copulation plug and pregnancy for 12 months. Among the eight randomly selected Sam68<sup>−/−</sup> females, two were sterile and did not yield litters, whereas the others gave birth to a reduced cumulative number of pups when compared with their WT littermates (13.1 versus 43.8; Fig. 1B). Subfertility of Sam68<sup>−/−</sup> females derived from at least three different components. First, they delivered their first litter nearly 2 months later than WT females (16.8 ± 2.7 versus 8.6 ± 2.4 weeks of age, P < 0.01; Fig. 1C). Secondly, they required an increased breeding time for successful mating (26 ± 6 days versus 5.7 ± 1.5, P < 0.01; Fig. 1D). Lastly, the mean number of pups per litter was also reduced in the KO females compared with WT mothers (4.9 ± 0.5 versus 8.2 ± 0.4, P < 0.01; Fig. 1E).

Reduced number of growing follicles in the ovaries of Sam68<sup>−/−</sup> females

To determine whether Sam68 expression was required for ovarian development and function, we analysed the morphology of ovaries of WT and KO females at different ages. Sam68<sup>−/−</sup> females have smaller ovaries compared with WT littermates at all ages examined (Fig. 2A). Moreover, the weight of WT ovaries nearly doubled from 4 to 16 weeks of age (from 3.3 ± 0.6 to 6.2 ± 0.7 mg), whereas KO ovaries showed only a small increase (from 2.2 ± 0.1 to 2.9 ± 0.7 mg) (Fig. 2B). Sam68<sup>−/−</sup> females display reduced body weight, which remains constant during ageing (Fig. 2C). Nevertheless, the ratio between ovary and body weight was significantly decreased (P < 0.01) at all ages tested, and the defect was more pronounced with ageing (Fig. 2D). These results indicate that the reduced ovarian weight is a specific phenotype of Sam68<sup>−/−</sup> females and not a reflection of their smaller size.

The increase in size and weight of the ovary after birth parallels the growth of developing follicles from the primordial to the antral stage (25). To determine whether ablation of Sam68 affected specific stages of follicular development, we performed accurate counts of growing follicles at different ages (see Materials and Methods and Fig. 3A). At 4 weeks after birth, despite the reduced dimension of the organ (Fig. 2B and D), the ovaries of KO and WT females showed a comparable number of follicle types (Fig. 3B). In contrast, the number of secondary and early antral follicles was significantly reduced in 8-week-old Sam68<sup>−/−</sup> females compared with the WT counterparts (P < 0.05; Fig. 3C). This reduction was maintained in older females of 16 weeks of age (P < 0.05;...
Fig. 3D), indicating that proper follicle development is not recovered with time. In contrast, neither primordial follicles, which represent the oocyte reservoir, nor primary follicles were significantly affected in the KO ovary.

Ablation of Sam68 results in irregular oestrous cycles and reduced hormonal response by the ovary

The transition from primordial to secondary follicles is gonadotropin independent and largely controlled by paracrine growth factors (17,26). In contrast, survival of secondary follicles and their growth to the antral stage depend on the establishment of a pituitary–gonadal axis, which coordinates secretion of gonadotropins with the production of oestrogens by the growing follicles and allows oestrous cycles to progress properly after puberty (20). Subfertility defects observed in adult Sam68<sup>−/−</sup> females and the reduced number of growing follicles suggested a possible impairment of the gonadotropin-dependent phase of folliculogenesis. Indeed, analysis of the oestrous cycle by vaginal smears showed that Sam68<sup>−/−</sup> adult females undergo an irregular alternation of phases, resulting in a significant reduction in the number of oestrous cycles (P < 0.05; Fig. 4A).

Next, we asked whether follicle maturation and ovulation could be recovered by providing exogenous gonadotropins to Sam68<sup>−/−</sup> females. Ovulation was induced by injection of pregnant mare serum gonadotropin (PMSG), which mimics FSH action, followed 44 h later by injection of hCG, which mimics LH action. Under these conditions, Sam68<sup>−/−</sup> females yielded approximately half of the oocytes obtained with stimulation of WT females (P < 0.05; Fig. 4B), indicating that Sam68<sup>−/−</sup> follicles responded less efficiently to the gonadotropin stimulus. Nevertheless, the fewer oocytes produced by hormonally induced Sam68<sup>−/−</sup> females were fertilized at the same rate as those of their WT littermates, and they efficiently progressed through pre-implantation development in vitro (Supplementary Material, Fig. S2A and B), reaching a normal blastocyst stage (Supplementary Material, Fig. S2Cu and c). These results indicate that the reduced number of pups delivered by Sam68<sup>−/−</sup> females is due to impaired hormone-dependent follicle development rather than defective early embryo development.

Sam68 supports the expression of the gonadotropin receptors in the ovary

The stimulation of follicle development by gonadotropins depends on the presence of specific receptors in the follicular cells. Notably, Sam68<sup>−/−</sup> mice exhibited disordered oestrous cycles, ovulatory defects and atrophic ovaries similar to the Fshr<sup>−/−</sup> mice, which display a much stronger infertile phenotype (27–29). This observation suggested that Sam68 might be implicated in the gonadotropin response of ovarian follicles. Expression of Fshr begins at the secondary follicle stage and sharply increases together with Lhcgr during the transition from early antral to the pre-ovulatory stage, rendering the follicle sensitive to the LH/FSH surge that drives ovulation. Given that the administration of exogenous gonadotropins could not restore normal ovulation in Sam68<sup>−/−</sup> females, we asked whether Sam68 ablation affected the expression of the FSH and LH receptors. Remarkably, quantitative real-time polymerase chain reaction (PCR) analyses indicated that both
Fshr and Lhcgr mRNAs were reduced in the ovaries of 8- and 16-week-old KO females (Fig. 5A). Since Sam68 regulates the metabolism of several mRNAs through a direct interaction (4,6–8,15,30,31), we also asked whether it could bind the Fshr and Lhcgr mRNAs. Crosslinking RNA/protein co-immunoprecipitation (CLIP) assay from WT and KO ovaries (Fig. 5B, left panel) followed by RT–PCR analysis of the covalently linked RNAs demonstrated that Sam68 directly binds to both Fshr and Lhcgr mRNAs (Fig. 5B, right panel). The interaction was specific because Sam68 did not bind to Mucin3, an mRNA not bound by this RBP in other tissues (14), nor to H2afz, an mRNA abundantly expressed in mouse ovary (32). Moreover, the Fshr and Lhcgr mRNAs were not immunoprecipitated in the absence of Sam68 from the KO ovary or by a control antibody (Fig. 5B). These results suggest that Sam68 might regulate Fshr and Lhcgr expression in the mouse ovary by binding to their mRNAs.

Sam68 expression is required during the gonadotropin-induced response of follicular cells

A sharp increase in the Fshr and Lhcgr mRNA levels occurs in follicles during the transition from the early antral to pre-ovulatory stage, due to both increased transcription and stabilization of these transcripts (33–35). To test whether Sam68 takes part to this gonadotropin-induced ovulatory response, we treated immature 21-day-old WT females, before the onset of their physiological oestrous cycles, with PMSG to promote the in vivo growth of early antral follicles. As expected, Fshr and Lhcgr mRNAs were upregulated in the ovary 8 h after PMSG injection, and their level remained high after 24 h (Fig. 6A). Remarkably, we observed that Sam68 mRNA levels were also increased, with a time course that appeared to precede (maximal at 8 h) that of the transcripts for the gonadotropin receptors (Fig. 6A), and it correlated with an increase in protein levels (Fig. 6B).

Next, we isolated follicular cells from early antral follicles present in ovaries of immature 21-day-old WT and KO females. Cells were treated with 8-Br-cAMP, an analogue of the second messenger cAMP that transduces the FSHR signal (36), to bypass the lower levels of receptor expressed by KO cells and to check whether the intracellular events activated by the hormones were affected in Sam68−/− follicular cells. Similar to the in vivo gonadotropin response, this treatment leads to upregulation of both the Fshr and Lhcgr mRNAs in WT follicular cells (35) after 24 h of treatment (Fig. 6C).
Interestingly, 8-Br-cAMP also strongly induced expression of Sam68, indicating that this RBP is directly involved in the FSH-dependent response of follicular cells (Fig. 6C). Furthermore, Sam68−/− follicular cells were dramatically impaired in this response, and the accumulation of the Fshr and Lhcgr mRNAs was abolished and strongly reduced, respectively (Fig. 6C). These results suggest that Sam68 takes part to the gonadotropin-induced differentiation of the pre-ovulatory follicle by allowing accumulation of the Fshr and Lhcgr mRNAs in follicular cells.

DISCUSSION

Sam68 is expressed in most tissues of the organism, and it is particularly abundant in the brain and in the male and female gonads (3). In line with its expression levels, mice ablated for this gene show defects in motor coordination (10), and males are infertile (15). Herein, we have investigated the role of Sam68 in the regulation of female fertility. Our study indicates that Sam68−/− females are subfertile and display a dramatic reduction in the cumulative number of pups delivered during their lifespan. Such fertility defects can be attributed to both delayed sexual maturity and permanent impairment of the oestrous cycle due to defective development of follicles in adult females.

The observation that KO females become fertile and deliver their first pups much later than their WT littermates can be explained by the described delayed maturation of sexual organs required for pregnancy and lactation in Sam68−/− females (11). Nevertheless, these defects were rescued with ageing, and they almost disappeared in 12-week-old animals (11). Thus, additional defects are responsible for subfertility of adult females. For instance, we observed that Sam68−/− females required a longer housing time with the male than WT littermates to become pregnant. Since this defect was also maintained after the first pregnancy, it was not due to the delay in sexual development. Moreover, KO mothers delivered a reduced number of pups throughout their entire life. These defects in adult animals suggested that ablation of Sam68 caused an imbalance in the hormonal axis that regulates the oestrous cycle and ovulation. In line with this hypothesis, we found that Sam68−/− females had extremely irregular oestrous cycles, which were only partially rescued by the administration of exogenous gonadotropins, indicating that a lack of pituitary hormones could not account for the phenotype. Interestingly, the irregular oestrous cycles, the ovulatory defects and the smaller size of the ovaries caused by Sam68 ablation resembled the phenotype of Fshr−/− females (28,29). These observations support the hypothesis that Sam68 is required in some steps of hormonal regulation of ovarian functions, downstream of the production of pituitary hormones.

Folliculogenesis is controlled by paracrine and endocrine factors that coordinate oocyte and follicle development (23,37,38). The early phases, from primordial to secondary follicles, are modulated by paracrine ovarian factors such as GDF9 and BMP15 produced by the oocyte itself (39). At puberty, the onset of release of pituitary gonadotropins insures follicle development from the early antral to the pre-ovulatory stage. This regulation is maintained in adult females, in which the recruitment for a cohort of immature

Figure 3. Growing follicles are reduced in Sam68−/− ovaries. (A) Histology of an ovarian section with indicated examples of the different follicle categories counted. Each group was scored in serially sectioned ovaries stained with haematoxylin/eosin as described (48) obtained from 4-week-old (B), 8-week-old (C) and 16-week-old (D) Sam68+/+ and Sam68−/− females. Statistical significance (∗P < 0.05) was calculated by Student’s t-test.
increased transcription and stability (34, 42). Our results now suggest that Sam68 takes part in this regulation. Notably, the effect of Sam68 on Fshr expression levels might also explain the similar defects in bone loss exhibited by Sam68 and Fshr KO females (9, 29). Osteoporosis is usually associated with hypogonadism and age-related menopause; however, ablation of either the FSH receptor or Sam68 suppressed bone loss in mouse models. It will be of interest to determine whether Sam68 regulates Fshr expression in bone cells similar to what we have observed in follicular cells.

The high complexity of the gonadotropin-induced response of ovarian follicles requires key regulatory steps that need to be finely tuned to insure female fertility (43). Indeed, many human syndromes that cause sterility involve a reduced sensitivity to hormone stimulation (44, 45), even though in many cases the genetic cause remains unknown. Our observations led to the unexpected finding that Sam68 occupies a relevant role in this intricate pathway involved in the follicular response to gonadotropins. Thus, it is possible that the aberrant expression or function of Sam68 underlies some cases of unexplained female subfertility that cannot be fully recovered by the administration of exogenous gonadotropins, such as premature ovarian failure (45), primary hypogonadism, polycystic ovarian syndromes (44) and gonadotropin-resistant ovary syndrome (46).

In conclusion, our study highlights a novel function for Sam68 in the complex network that regulates the expression of the gonadotropin receptors during follicle development and suggests that defects in such protein can strongly reduce follicle maturation and ovulation, thereby limiting the female fertility potential.

MATERIALS AND METHODS

Breeding assay

Mice were maintained by intercrosses of Sam68+/− mice as described previously (9). Four-week-old Sam68+/+ and Sam68−/− females were individually housed with heterozygous males of proven fertility and monitored for pregnancy continuously for 1 year. The number of pups delivered was checked at birth and at the time of weaning, when the mothers were mated with another male. The number of mating days was considered as the number of days passed by the females together with the males before they get pregnant. The first mating was not included in this score.

Oestrous cycle analysis and quantification of follicles

The oestrous cycle was monitored for 20 days in adult females (n = 5), and the stages of the cycle were determined by analysis of the vaginal smears. The follicle classification system used was based on published protocols (47, 48). Briefly, follicles were scored as primordial when the oocyte was surrounded by flattened granulosa cells, primary when the oocyte was surrounded by one layer of cuboidal follicular cells, secondary when the oocyte was surrounded by two to three layers of cuboidal follicular cells, early antral when the oocyte was surrounded by more than three layers of cuboidal follicular cells and antral when they contained a single large antral cavity. A minimum of four ovaries was collected for

Figure 4. Sam68 ablation leads to irregular oestrous cycles and affects the hormonal response in the ovary. (A) Analysis of the oestrous cycle by vaginal smears for 20 consecutive days. Graph of the mean ± SD of the number of oestrous cycles in younger and older females. (B) Graph of the oocytes ovulated after injection of exogenous hormones (PMSG and hCG) in WT and KO females. Data represent the average number of oocytes collected from superovulated Sam68+/+ and Sam68−/− mice (n = 4). Statistical significance (*P < 0.05) was calculated by Student’s t-test.
each genotype from 4-, 8- and 16-week-old mice. Ovaries were serially sectioned (8 μm), stained with haematoxylin and eosin and analysed for the number of healthy follicles. Repetitive counting of the same follicles in every fifth section through the entire ovary was avoided by counting only follicles containing oocytes with a visible nucleolus.

**Immunohistochemistry**

Ovaries were fixed overnight in Bouin’s solution at 4°C and embedded in paraffin. Longitudinal sections of about 5 μm were mounted on StarFrost-Plus slides (Knittel-Glaser, Germany) and processed using standard protocols. Antigen retrieval was achieved by heating treatment in 1 mM EDTA. Sections were incubated with the α-Sam68 (sc-333, Santa Cruz Biotechnology, U.S.A.) primary antibody and with the horseradish peroxidase-conjugated secondary antibody. Chromogenic detection was performed following the manufacturer’s instructions (UltraVision One Detection system, Thermo Scientific, U.S.A.).

**Measurement of ovulation and in vitro development**

MII oocytes and one-cell embryos were collected from hormonally primed adult female mice injected with 5 IU of PMSG and 5 IU of hCG (Intervet) 48 h later and freed of cumulus cells by a brief treatment with hyaluronidase (500 μg/ml) in M2 medium (Chemicon, U.S.A.). In vitro development was followed by culturing zygotes in potassium simplex optimized medium (Chemicon) under mineral oil for 5 days (49). Immunofluorescence staining of the blastocysts was performed as described previously (24).

**Statistical analysis**

Data are reported as mean ± SD, and each sample represents at least four independent observations. Differences between groups of WT and KO animals were analysed statistically. Student’s t-test was applied for direct comparisons, whereas one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison test was used for multiple testing between groups. P-values less than 0.05 were considered significant and are indicated with an asterisk in the figures. When present, higher significant differences (P < 0.01) are also specified in the text.

**UV crosslinking and RNA immunoprecipitation**

Partially dissociated ovaries from WT and KO mice were irradiated with 254 nm ultraviolet light (3 × 100 mJ/cm² on ice) using a Stratalinker, pelleted and snap frozen in liquid nitrogen (50). Tissue was resuspended in the lysis buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 1% NP40, 0.5% Na deoxycholate, 0.1% sodium dodecyl sulphate), supplemented with 30 U of RNase OUT (Invitrogen, U.S.A.) and incubated overnight with antibody-conjugated beads at 4°C. Immunocomplexes were treated with 50 U of DNase (Roche, U.S.A.) and subsequently with 50 μg of proteinase K before phenol/chloroform extraction of co-precipitated RNA. After reverse transcription, samples were used for PCR. Primers used for Mucin have been published previously.
amplification using the primers listed in Supplementary Material (Invitrogen) after DNase treatment and used for PCR.

RNA was extracted and reverse transcribed with Superscript II Hprt mRNA for 8 and 24 h to induce ovulation. (first panel), Fshr expression analysis was used as internal control of the amplification and integrity of the RNA extracted. (second panel) and Sam68 mRNA levels in WT and KO cells. Hprt mRNA analysis was used as internal control. (C) Treatment of follicular cells with 8-BrcAMP to mimic the activation of the gonadotropin receptor pathway. RT–PCR analysis of Fshr, Lhcgr and Sam68 mRNA levels in WT and KO cells. Hprt mRNA analysis was used as internal control. (D) Hypothetical model of the function of Sam68 in the gonadotropin response of follicular cells.

(14). Primers used for Fshr, Lhcgr and H2afz are listed in Supplementary Material, Table S1.

Real-time PCR analysis

Samples were harvested in TRIzol Reagent (Invitrogen) for RNA extraction. Reverse transcription was performed using M-MLV (Invitrogen), and quantitative real-time PCR was performed in triplicate using iQ Sybr-green Supermix (Bio-Rad Laboratories, U.S.A.) as described previously (51). 18S RNA was used to obtain the ΔCt values for the calculation of fold increases. Primers used for 18S are listed in Supplementary Material, Table S1.

Treatment of follicular cells with 8-Br-cAMP

Follicular cells from antral follicles were obtained by puncturing ovaries from 21-day-old mice and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum, with or without 1 mM 8-Br-cAMP for 24 h. RNA was extracted and reverse transcribed with Superscript III (Invitrogen) after DNase treatment and used for PCR amplification using the primers listed in Supplementary Material, Table S1.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We wish to thank Prof. Stéphane Richard for the generous gift of the Sam68 knockout strain used in this study and for sharing his observations on the phenotype of this strain. We also wish to thank Dr Susanna Dolci for helpful suggestions throughout the study and for discussion of the results, Dr Costanzo Moretti for discussion on human ovarian syndromes, Dr Francesca Klinger for assistance with estrous cycle analysis and Drs Valeria Messina and Alessia Latina for help with genotype analyses.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by grants from Telethon, Associazione Italiana Ricerca sul Cancro (AIRC) and Ministry of Education (PRIN 2006).

REFERENCES


