Mage-b4, a Novel Melanoma Antigen (MAGE) Gene Specifically Expressed during Germ Cell Differentiation

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ABSTRACT

The MAGE genes were initially isolated from different kinds of tumors, and on the basis of their specific expression in adult tissues, they have been used as targets for cancer immunotherapy. However, although a large number of MAGE genes have now been identified and extensively studied in tumors of various origins, their functions in normal cells remain unknown. Here we describe the isolation and characterization of a novel murine MAGE homologue, Mage-b4. mRNA expression studies in a wide variety of adult and embryonic tissues revealed that Mage-b4 is specifically expressed in fetal and adult gonads. An antibody specific to Mage-b4 was developed, and using this antibody, we found that the Mage-b4 protein was confined to the cytoplasm of germ cells. Double-labeling experiments using antibodies against the meiosis-specific SCP3 protein and the Mage-b4 protein showed that Mage-b4 is down-regulated as the germ cells enter meiosis in adult testis. In contrast, Mage-b4 was expressed in female germ cells throughout meiosis, and the protein was also found in dormant primary oocytes.

INTRODUCTION

In 1991, van der Bruggen et al. (1) identified the first melanoma antigen gene, MAGE-1 (MAGE-A1), in a human melanoma cell line. Since then, a growing number of MAGE genes have been isolated and shown to be expressed in a wide variety of tumors of different histological origin. This tumor-specific expression made the MAGE genes interesting as candidates for antitumor immunotherapy, and it was recently shown that injection of MAGE-3.A1 peptide into patients with metastatic melanoma induced tumor regression (2, 3). Furthermore, it was shown that MAGE-A1 can also functionally replace the Rb as a growth suppressor in Rb-deficient SAOS-2 osteosarcoma cells, which suggests that necdin is a neuron-specific growth suppressor with a function similar to that of Rb (19).

In the present study, we describe the isolation and the expression pattern of a novel murine gene, Mage-b4, which shows significant homology to the human MAGE-B genes. Mage-b4 is exclusively expressed in germ cells, and in adult testis is restricted to pre-pachytene cells. In contrast, female germ cells show a wider distribution of Mage-b4, with expression in both premeiotic germ cells and germ cells that have gone through the pachytene phase and entered meiotic arrest.

MATERIALS AND METHODS

Screening of Fetal Testis cDNA Library. Primers specific for MAGE-B1 (Mage-5*, 5’-AGG AAT GGG CCT CTG ATG CC-3’; and Mage-3*, 5’-CTC TTC ATA ATG GGA TGG GAA-3’) were used to PCR amplify cDNA from 14.5 dpc testis. Reverse transcription and PCR reactions were performed in the same way as described previously for semiquantitative RT-PCR (20) except that the numbers of PCR cycles were increased to 30. A DNA band of 330 bp was isolated and sequenced. This DNA was then used as a probe to screen a fetal testis cDNA library (constructed with the ZAP Express cDNA synthesis kit; Stratagen). The screening was carried out according to the manual for the ZAP Express cDNA synthesis kit (Stratagen). A total of 2.5 × 10^6 recombinants were screened, and ~10 positives were found. From these, three cDNAs clones were isolated; termed 4.1, 4.2, and 4.3.

Sequencing. DNA was sequenced using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer), and the samples then were processed on a 373 A automated DNA sequencer (Applied Biosystems).

RNA Extraction. Total RNA from male and female gonads with attached mesonephros was isolated from mouse embryos at 11.5, 12.5, 13.5, 14.5, 15.5, 16.5, and 17.5 dpc and treated with DNase to remove contaminating DNA as described previously (20).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as described previously (21). The following primers were used: Hprt-5* (5’-CCT GCT GGA TTA CAT TAA AGC ACT G-3); Hprt-3* (5’-GTC AAG GGC GCT GGA TTA CAT TAA AGC ACT G-3); Mage-5*, Mage-3* (described above).

Immunoblotting. Immunoblotting was performed as described previously (22) with the following modifications: tests and ovary from adult mice were lysed in SDS-reducing buffer. In vitro-translated proteins were made using the TNT T3 Coupled Reticulocyte Lysate System (Promega), with or without the Mage-b4-containing plasmid 4.1. Protein extracts were separated using 12% SDS-PAGE and transferred to an Immobilon-P filter. The filter was blocked.

Received 8/13/99; accepted 12/15/99.
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1 Supported by the Jeansson’s Foundation, Åke Wiberg’s Foundation, Magn. Bergvall’s Foundation, and the Swedish Cancer Foundation (4100-B98-01XAB).

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with 5% milk powder in PBS with 0.1% Tween. The primary antiserum, anti-Mage-b4, was diluted 1:100.

**Plasmid Constructs and Transfections.** The full-length Mage-b4 sequence from plasmid 4.1 was cloned into the eukaryotic expression vector pBK-CMVlac, and the plasmid was named pCMV-Mage-b4. CHO cells were transfected with pBK-CMVlac or pCMV-Mage-b4, using the LIPO-FECTAMINE Reagent (Life Technologies, Inc.). Immunofluorescence was measured as described below except that the cells were fixed in 95% methanol in PBS for 5 min.

**In Situ Hybridization.** For the Mage-b4 riboprobe, digoxigenin-labeled antisense and sense RNA probes were prepared from T7-promoter-containing PCR products as described previously (21). The Mage-b4 3′ primer was as follows: 5′-TTT ATG ACT CCA TCT ACT GTA GGG CAG CTG ATA ACA ACA ACT GAG TA-3′, where the T7 promoter sequence is shown in bold. The Mage-b4 5′ primer was 5′-GGC AGA TAT GTC TTT AGG TT-3′.

**Antibody Production.** Rabbit antisera to Mage-b4 were made using a 15-amino acid synthetic peptide (CDKQAESKVTLDSS), corresponding to one of the repeats in the COOH-terminal part of the predicted Mage-b4 protein.

**Immunohistochemistry.** Embryonic male and female gonads, 13.5, 14.5, 15.5, and 17.5 dpc, and adult testes and ovaries from mouse were fixed in 1% paraformaldehyde in PBS for 1 h on ice, and then equilibrated in 0.5 M sucrose for 1 h on ice and embedded in OCT compound (Miles) before being frozen and cryosectioned to 7-μm sections. Immunofluorescence was performed as described previously (21). The primary polyclonal Mage-b4 antiserum was diluted 1:100.

**Double Immunohistochemistry.** Adult testes from mouse were fixed, embedded, and sectioned as described above and kept at −20°C. The sections were moved to room temperature and treated for 10 min with 0.2% Triton X-100 in PBS, followed by blocking in 3% BSA in PBS for 30 min. The sections were then incubated with the first primary antibody (rabbit anti-SCP3, diluted 1:10 in blocking solution) for 40 min, washed in PBS four times (10 min for each wash), and then incubated with the first secondary antibody (swine antirabbit rhodamine-conjugated antibody, diluted 1:100 in blocking solution) for 90 min. After being washed in PBS four times (10 min for each wash), the sections were incubated with the second primary antibody (rabbit anti-Mage-b4, diluted 1:100 in blocking solution) for 40 min, washed three times in PBS (10 min for each wash), and then incubated with the second secondary antibody (swine antirabbit FITC-conjugated antibody, diluted 1:100 in blocking solution) for 20 min. The sections were washed twice in PBS (10 min for each wash), stained with a solution of Hoechst 33258 (1 μg/ml) for 1 min, and mounted in a 78% glycerol mounting medium (containing 1 mg/ml p-phenylendiamine).

**Databases Used for Protein Predictions.** Several databases were used for protein predictions: TMpred (www.ch.embnet.org/software/TMpred_form.html) for membrane-spanning regions; GCG/SqWeb (dbm.cgr.se:8010/ncg-bin/sqweb.cgi) for isoelectric points; SignalP (www.cbs.dtu.dk/services/SignalP) for predicted signal peptide; and Caenorhabditis elegans (www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml) and yeast (ales.med.umn.edu/gbsearch/ybp2.html) sequences for homology searching, using BLAST.

**RESULTS**

**A Novel Mouse Gene Related to the Human MAGE-B Genes.** To isolate a mouse homologue to the human MAGE-B genes, MAGE-B1-specific PCR primers were used to PCR amplify fetal testis cDNA, and a 330-bp DNA fragment was isolated and sequenced. This DNA fragment had a high sequence homology to the MAGE-B1 gene (data not shown) and was then used as a probe to screen a mouse 13.5 dpc fetal testis cDNA library. Three overlapping clones were identified, termed 4.1, 4.2, and 4.3, which were 2204, 2089, and 1327 bp in length, respectively (Fig. 1A). One ORF was identified that encodes a putative protein having 468 amino acids. Multiple stop codons were present before and after the coding sequence (Fig. 1A, marked with *), and a putative polyadenylation signal was located 11 bp upstream of the poly(A) stretch. The NH2-terminal part of the amino acid sequence derived from the ORF was similar to that of the MAGE proteins, with highest homology (~55% identity) to the human MAGE-B proteins, and moderate homology (~47% identity) to the three previously characterized mouse MAGE-b homologues, MAGE-b1 to -b3 [also named Smage (1 to 3); Fig. 1B; Table 1]. The Mage-b4 protein showed the same homology as Mage-b1 to the human MAGE-B proteins (~55% identity; data not shown), indicating that different amino acids have been conserved between Mage-b4, Mage-b1, and the human MAGE-B proteins. The COOH-terminal part of the ORF is ~130 residues longer than the other Mage-b proteins because of an insertion of an unique repetitive sequence. The repetitive part of the ORF is unrelated to any sequence in the EMBL/GenBank databases. It consists of 15 amino acids, almost perfectly repeated nine times (399 bp, 133 amino acids; Fig. 1A, marked with brackets).

The predicted protein from clones 4.1–4.3 can be divided into four domains: the NH2-terminal end domain, amino acids 1–16, which is highly homologous to the protein encoded by the human MAGE-B gene (94% identity); a second domain, amino acids 17–197, with lower homology (37% identity); a third domain, amino acids 198–320, which is more highly conserved (73% identity); and the final domain, amino acids 320–468, which has no equivalent in the human MAGE-B genes and includes the repetitive sequence (Fig. 1C). Because this new mouse gene showed highest homology to the human MAGE-B genes, it was named Mage-b4, after the previously identified mouse Mage-b1/2 and Mage-b3 genes.

**Mage-b4 Is Expressed in Fetal Gonads and Adult Testis.** We next examined the expression of Mage-b4 in adult and fetal organs and during mouse gonad development, using semiquantitative RT-PCR. The expression pattern was very restricted, and we detected the mRNA only in fetal gonads and adult testis (Fig. 2A). The expression of mRNA started between 13.5 and 14.5 dpc in both XX and XY gonads. The expression in XX gonads then stayed at approximately the same level throughout fetal development, whereas in XY gonads, Mage-b4 mRNA levels increased and remained high just before birth (Fig. 2B).

**Identification of the Mage-b4 Protein.** To make a preliminary analysis of the Mage-b4 protein, a polyclonal antibody serum was developed against the unique repetitive peptide in the COOH-terminal end of Mage-b4. The polyclonal serum was used on a Western blot to analyze protein extract from adult testis, ovary, and in vitro translated Mage-b4 protein (Fig. 3A). In vitro transcription/translation of the Mage-b4 cDNA yielded a protein that migrated at ~70 kDa on an SDS-polyacrylamide gel. When we used samples from adult testis, a major protein band of ~80 kDa appeared. A faint band was observed in the ovary after longer exposure. No bands were observed when reticulocyte lysate without Mage-b4 RNA was used or when extracts from 3T3 and CHO cells (data not shown) were used. The molecular mass for Mage-b4 was higher than that predicted from the amino acid sequence (52 kDa). This discrepancy may be caused by unusual migratory properties of Mage-b4 in the SDS-PAGE. The same phenomenon has also been observed for other MAGE and MAGE-related proteins (18, 23–25). De Plaen et al. (13) have pointed out that there are remarkable differences in the isoelectric points (pI) of MAGE-A and MAGE-B proteins. MAGE-A proteins are acidic (pI 4–4.6), whereas MAGE-B proteins are basic (pI 9–10.7). Computer analysis of the predicted Mage-b4 protein gave a pI of 10.1, suggesting that the Mage-b4 protein, like human MAGE-B, is a basic protein. This could explain in part the peculiar migration properties during SDS-PAGE analysis.

We also used other computer programs to calculate the solubility of the protein, to look for signal peptides, and to look for membrane-spanning domains (see “Materials and Methods”). The results should be viewed with caution, but they may give some insight into the
characteristics of the protein. According to these analyses, the Mage-b4 protein is soluble and contains no signal peptide. A possible hydrophobic transmembrane domain, which has been shown to be conserved across the MAGE family, including the MAGE-B proteins (8), was observed at amino acids 192–216. The predicted Mage-b4 protein also harbors a potential N-glycosylation site (boxed region in Fig. 1A), and it is therefore possible that the protein found in adult testis contains a post-translational modification not present in the in vitro-translated protein, resulting in slower migration.

To verify the specificity of the serum further, the Mage-b4 antibody...
A detailed analysis of a protein’s expression pattern is usually the first step toward the dissection of its function. The in situ hybridization and immunohistochemistry results showed that Mage-b4 expression was restricted to the germ cells in fetal testis; therefore, a more careful study of Mage-b4 protein expression was carried out during germ cell differentiation in both male and female gonads. During embryogenesis, the primordial germ cells, which migrate from the base of the allantois via the hindgut and the mesonephros, colonize the fetal gonads. They proliferate as they migrate, and by 13.0 dpc in the mouse, each gonad contains ~10,000 germ cells (reviewed in Refs. 28–30). In the male gonad, the germ cells continue to proliferate for a few days and then arrest in the G2–G1 phase of the cell cycle. During this period, they become enclosed by differentiating Sertoli cells, forming the seminiferous cords. The germ cells are now renamed gonocytes. Shortly after birth, the gonocytes resume proliferation to give spermatogonia, which at puberty further differentiate into spermatocytes. These enter meiosis and subsequently are transformed into spermatids as a result of spermiogenesis. In the female gonad, meiosis is induced during the fetal stage. At 13.5 dpc, the first sign that germ cells are entering meiosis is observed, and by 16 dpc most of the primary oocytes are in the pachytene stage. After birth, the female germ cells enter meiotic arrest until puberty, at which point individual oocytes resume gametogenesis in response to surges of gonadotropic hormones.

During fetal development, Mage-b4 protein expression closely follows the pattern of RNA expression (Figs. 2 and 4). The protein appears in both XX and XY gonads between 13.5 and 14.5 dpc; it then increases in the male gonad and is heavily expressed in all male germ cells just before birth. In female gonads, Mage-b4 remains at an approximately constant level from 14.5 dpc until birth. It is clear that the Mage-b4 protein is located in the cytoplasm of germ cells in both female and male gonads throughout the fetal period (Fig. 4).

The different developmental stages of germ cells are well defined in the adult testis; therefore, Mage-b4 expression was investigated during germ cell differentiation in more detail in this tissue. The Mage-b4-specific antibody preferentially stained the cytoplasm of germ cells located close to the basal lamina of the seminiferous tubuli (Fig. 5A).

As expected, no signal was found in the Sertoli or interstitial Leydig cells. At least two different kinds of Mage-b4-positive cells are present (Fig. 5C). One type of positive cells has a large, uncondensed nucleus as shown by Hoechst staining (marked with * in Fig. 5, C and D). Hoechst 33258 is a DNA-binding dye that preferentially stains heterochromatic DNA (31). The cytoplasm of these cells is spread out and shows very strong Mage-b4 expression. The second type of Mage-b4-positive cells is smaller, rounder, and has a nucleus with condensed chromosomes (marked with # in Fig. 5, C and D). These cells show a weaker signal for Mage-b4. A careful examination of the Mage-b4-positive cells suggested that they are spermatogonia and early spermatocytes.

Several markers for the first meiotic prophase are available, including a component, SCP3, of the synaptonemal complex (reviewed in Ref. 32). An antibody toward SCP3 and the Mage-b4-specific antibody were used in double immunohistochemistry experiments to map in more detail Mage-b4 expression during male meiosis. SCP3 appears in the nucleoli of early meiotic cells; the protein then reorganizes along the chromosomes and is seen as thin fibers during leptotene/zygotene. In pachytene cells, the SCP3-specific antibody recognizes ~20 clearly visible fibers that constitute the paired chromosomes (22). Mage-b4 expression was high in spermatogonia. Its level then diminished during the transition into early meiosis, and no protein could be detected in cells that had entered the pachytene stage (Fig. 6A).

Mage-b4 Is Expressed in Female Germ Cells during the Pachytene Phase. In the ovary, germ cells are in the pachytene stage between 14 dpc and day 2 after birth (33). The expression pattern of Mage-b4 during female fetal development suggested that Mage-b4 levels remain constant throughout this period and that the protein might be expressed in pachytene cells (Fig. 4). This was confirmed with double immunohistochemistry using the SCP3 and the Mage-b4 antibodies (Fig. 6, B–E). We next examined Mage-b4 expression when the female germ cells had gone through pachytene and entered meiotic arrest, at days 2–8 after birth (Fig. 6, F–J). An antibody to c-kit was used as a positive control because this protein has been shown to be expressed on the cell surface of all oocytes at this stage.

### Table 1 Amino acid identities and similarities between Mage-b4 protein (amino acids 1–320, the repetitive sequence excluded) and some of the human and mouse MAGE homologues

<table>
<thead>
<tr>
<th>MAGE Homologue</th>
<th>Mage-b4 Identity (%)</th>
<th>Mage-b4 Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-B1 (Xp)</td>
<td>54</td>
<td>61</td>
</tr>
<tr>
<td>MAGE-B2</td>
<td>56</td>
<td>61</td>
</tr>
<tr>
<td>MAGE-B3</td>
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<td>54</td>
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<tr>
<td>MAGE-B4</td>
<td>56</td>
<td>62</td>
</tr>
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<tr>
<td>MAGE-A10</td>
<td>39</td>
<td>48</td>
</tr>
<tr>
<td>Mage-b1/2</td>
<td>47</td>
<td>55</td>
</tr>
<tr>
<td>Mage-a5</td>
<td>36</td>
<td>46</td>
</tr>
<tr>
<td>Necdin (mouse)</td>
<td>31</td>
<td>40</td>
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Amino acid identities and similarities between Mage-b4 protein (amino acids 1–320, the repetitive sequence excluded) and some of the human and mouse MAGE homologues.
Mage-b4 showed the same expression pattern as c-kit, i.e., in oocytes in both primordial follicles, located peripherally in the ovary, and in growing follicles, located more centrally. The Mage-b4 signal was weaker in growing oocytes; whether this is due to lower expression or to dilution related to the enlargement of the cytoplasm is unknown at present. Very few Mage-b4-positive cells were detected in the adult ovary, and these positive cells were always located close to the surface of the ovary (Fig. 6, K and L).

**DISCUSSION**

We have identified a novel murine gene, Mage-b4, by screening a fetal testis cDNA library. Most MAGE genes isolated thus far have been found because of their expression in tumor cells. However, some experiments have indicated that these genes may be expressed in embryonic tissues, for example, expression of Mage-a and Mage-b genes has been detected in blastocysts and ES cells (12, 13). A detailed analysis of the expression of Mage-b4 during the fetal period showed that Mage-b4 expression is restricted to the gonads, and more specifically to the germ cells.

Searches for homology with known sequences in the EMBL/GenBank databases showed that Mage-b4 has a high homology to the human MAGE-B genes and slightly lower homology to the murine Mage-b genes. Phylogenetic analysis using the neighbor-joining method placed the Mage-b4 gene between Mage-b1/2/3 and the human MAGE-B genes (data not shown). On the basis of sequence homology and comparison of isoelectric points, we suggest that Mage-b4 is a murine homologue of the MAGE-B genes. Southern blot analysis showed that the Mage-b4 gene is located on the X chromosome (data not shown), but a precise location has not yet been determined. It will be interesting to see whether the gene maps to the Mage-b1/2/3 cluster or somewhere else on the X chromosome (12). Regions of the predicted Mage-b4 protein that are most homologous to the corresponding regions in the MAGE-B proteins include amino acids 1–16 and 199–320. It is likely that important functions for the protein reside in these regions because they have been conserved during evolution. It is also possible that the specificity for the different Mage genes is determined by the less conserved region. Thus far, no specific function has been attributed to any region of the MAGE proteins.

Mage-b4 has an unique feature that most MAGE proteins do not have. In addition to its 320-amino acid MAGE-homologous part, it has a repetitive region consisting of 133 amino acids at the COOH-terminal end. We have also looked for Mage-related genes in yeast and C. elegans, without success, suggesting that the Mage genes have evolved rapidly or late.

The Mage-b4 gene has a very specific expression pattern during germ cell development, which strongly suggests that it has an essential role during gametogenesis. The process of mammalian gametogenesis differs greatly in timing between the female and the male. In the mouse, female germ cells enter meiosis during the fetal period, whereas in the male, the meiotic phase is initiated just before puberty. In both systems, Mage-b4 expression is confined to the cytoplasm of premeiotic cells. As soon as the cells enter the meiotic phase in the adult testis, Mage-b4 expression is down-regulated, and in the pachytene stage, no Mage-b4 protein could be detected. This expression pattern is compatible with at least two possible functions for Mage-b4 in adult testis. First, this gene could be important for keeping
Fig. 4. Expression of Mage-b4 protein during fetal gonad development, from 13.5 to 18.5 dpc, using immunofluorescent staining with the Mage-b4-specific antibody. Chromatin is stained with Hoechst.

Fig. 5. Expression of Mage-b4 protein in adult testis. A, immunostaining of Mage-b4 protein in mouse adult testis. B, the section shown in A, stained with Hoechst. C, close up of Mage-b4-positive cells, showing two different cell types, marked with * and #. D, the section shown in C, stained with Hoechst.
the germ cells in an undifferentiated stage. When the Mage-b4 protein is removed, the germ cells are allowed to enter the first meiotic division. This idea is in line with the function of the MAGE-related protein necdin, which acts as a growth suppressor in postmitotic neurons. A second possibility involves Mage-b4 in germ cell-specific mitosis.

In contrast to the restricted expression of Mage-b4 in adult testis, female germ cells show a wider distribution of Mage-b4, with expression in both premeiotic germ cells and germ cells that have gone through the pachytene phase and entered meiotic arrest. These differences in expression patterns may reflect the differences between the two sexes in the mechanism underlying the regulation of meiosis. Not only the timing is different in male and female meiosis, the requirement for cell cycle proteins is also different. For example, male mice lacking the cyclin A1 protein are sterile because of a block in spermatogenesis, whereas female mice lacking this protein show no defects in oogenesis (35). Thus, cyclin A1 has an essential role for the entry of male germ cells into the first meiotic division, whereas it is less important for the meiotic cycle of female germ cells. In addition, male mice lacking Hsp70-2, CREM, or A-myb show impaired fertility due to failures in different steps of germ cell development (36–38). Female mice lacking these genes show normal germ cell development and are fertile. It is possible that Mage-b4 belongs to this group of genes, which are required for male but not for female germ cell development.

It previously has been shown that most murine and human MAGE genes are expressed in the adult testis. Both MAGE-A1 and MAGE-A4 proteins have been detected in the nucleus and in the cytoplasm of spermatogonia and spermatocytes (14), and the Mage-b1/2/3 genes are expressed in postmeiotic germ cells (15). However, to our knowledge, this report represents the first example of a careful expression study of a MAGE-related gene during germ cell differentiation, from fetal to adult life and in both female and male gonads. It will be interesting to see whether other MAGE proteins also are expressed in a restricted pattern during the gametogenic process. If that is so, the MAGE proteins may be a new family of proteins involved in germ cell differentiation.

ACKNOWLEDGMENTS

We thank Eva Brundell and Christer Höög for protein lysate and the SCP3-specific antibody. We also thank Christer Höög, Yuan Li, and Karin Schmekel for stimulating discussions and helpful comments on the manuscript.

REFERENCES


![Fig. 6. Expression of Mage-b4 protein during male and female germ cell development. A, double immunostaining of SCP3 (red) and Mage-b4 (green) in adult mouse testis. Pachytene (green) and high (blue) in adult ovary at low (K) and high (L) magnification.](image-url)


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