The mouse Mageb18 gene encodes a ubiquitously expressed type I MAGE protein and regulates cell proliferation and apoptosis in melanoma B16-F0 cells

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INTRODUCTION

To date, more than 120 MAGE (melanoma antigen) genes and pseudogenes have been identified in the human, mouse and rat genomes [1]. On the basis of the difference in sequence similarity and chromosome location, the MAGE genes are divided into several subfamilies, including three acidic, MAGEA, MAGEB and MAGEC, one basic, MAGED, and others [MAGEE, MAGEF, MAGEG, MAGEH, NDN (necdin) and NDNL2 (NDN-like 2)]. They can also be classified into type I or type II genes on the basis of their expression pattern and functions. The type I MAGE genes are composed of the MAGEA, MAGEB and MAGEC subfamilies, whereas the other subfamilies belong to the type II MAGE genes [2,3]. Type I MAGE genes have been found to be expressed in many different tumours, but their expression in normal tissues is restricted to germline tissues such as placenta, ovary and testis, which express small amounts of HLA molecules [4,5]. In contrast, type II MAGE genes are expressed ubiquitously in somatic cells of different tissues [6–9]. These unique expression properties highlight the type I MAGE genes as superior candidates for tumour immunotherapy.

In early 1994, one study first reported that a MAGEA1-specific CTL line derived from the tumour-infiltrating lymphocytes of a melanoma patient could exhibit antigen-specific MHC-class-I-restricted lysis of HLA-A1-bearing MAGEA1+ cell lines in vitro [10]. The finding opened the possibility of immunizing HLA-A1 patients whose tumour expresses MAGEA1 either with the antigenic peptide or with autologous antigen-presenting cells pulsed with the peptide. A phase I/II study showed that vaccination of advanced cancer patients with MAGEA3 self-antigen indeed elicited MAGEA3-specific antibodies and a T-cell response [11]. These immune responses were also observed in several studies performed in patients with head and neck cancer [12], multiple myeloma [13], colorectal cancer [14], gastrointestinal carcinomas [15] and breast cancer [16].

The studies described above indicated that expression of type I MAGE genes were restricted to germ cells in normal tissues and to different malignancies; however, in our attempt to screen the expression profile of all mouse type I MAGE genes using the EST (expressed sequence tag) database in GenBank®, we found that, although most of the mouse ESTs identified were cloned from cDNA libraries that contained mouse testis, ovary or embryonic tissues, three of these ESTs (GenBank® accession numbers BY725125.1, BB327592.2 and BB645562.1) were cloned from cerebellum or adipose tissues. Moreover, these three ESTs reflected the same gene, Mageb18 (melanoma antigen family B 18). These results led us to assume that the expression of certain type I MAGE genes was not testis-specific, but ubiquitous in normal tissues.

To address this hypothesis, we sought to study the expression and function of type I MAGE genes using mouse Mageb18 as a model. To this end, we have demonstrated that Mageb18 is a type I MAGE gene conserved in higher mammals. It is expressed in digestion- and immune-related tissues as well as testis. Moreover, Mageb18 is also expressed in many mouse-derived cell lines, and both DNA demethylation and histone acetylation mediate the reactivation of Mageb18 in Mageb18-negative cell lines. Meanwhile, Mageb18 plays important roles in regulating cell

Key words: apoptosis, cancer immunotherapy, epigenetic regulation, expression pattern, melanoma antigen family B 18 (Mageb18), proliferation, subcellular localization.
proliferation and apoptosis. The results of the present study thus reveal an important phenomenon that the expression of some type I MAGE genes, at least for Mageb18, is not testis-specific, but ubiquitous. These findings also suggest that studying the expression and functions of MAGE genes in both normal tissues and tumours is very important for developing more effective and safer cancer vaccines.

**EXPERIMENTAL**

**Mice and ethics statement**

Female C57BL/6J mice (7–8 weeks old) were purchased from the laboratory animal centre of the Academy of Military Medical Sciences, Beijing, China. Maintenance of mice and experimental procedures were approved by the Animal Welfare and Research Ethics Committee of Nankai University.

**Cell lines and cell culture**

Mouse-derived cell lines used for expression analysis included H22, C6, B16-F0, 4T1, MM45TLi, L929, NIH 3T3 and RAW264.7. COS7 and HEK (human embryonic kidney)-293 cells were used to analyse the subcellular localization and overexpress the HA (haemagglutinin)-tagged MAGEB18 protein respectively. All of the cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine and 10% FBS (fetal bovine serum) in a 5% CO2 atmosphere at 37°C.

To study the influence of DNA methylation and histone acetylation on Mageb18 expression, H22 and C6 cells were treated with the DNA methylase inhibitor 5-aza-CdR (5-aza-deoxycytidine; Sigma–Aldrich) (1 μM) from day 1 to day 4. Meanwhile, the HDAC (histone deacetylase) inhibitor TSA (trichostatin A; Sigma–Aldrich) was added to the cells on day 3 at a concentration of 0.5 μM. Cells treated with either 5-aza-dC or TSA, or both, were harvested on day 4.

**Database searching and bioinformatics analysis**

In our preliminary studies, we have identified three ESTs (GenBank® accession numbers BY725125.1, BB327259.2 and BB645562.1) which were cloned from cerebellum or adipose tissues and reflected the same gene, Mageb18. To further characterize the expression and functions of Mageb18, a systematic sequence analysis strategy was first used. Briefly, the BLAT programs were used to determine the structure and relative position of the Mageb18 gene in the genome through the Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat). The Compute pl/Mw program (http://web.expasy.org/compute_pi/) was used to predict the pi and molecular mass. The subcellular localization was predicted using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/). N-linked glycosylation sites and phosphorylation sites were predicted using Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Multiple sequence alignments were carried out using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The neighbour-joining method was used to construct a phylogenetic tree by calculating the proportion of amino acid differences (p-distance), and the reliability of each branch was tested by 1000 bootstrap replications. To find potential homologues of the Mageb18 gene, mouse full-length MAGEB18 protein was used as a query to search the NCBI GenBank® non-redundant protein sequences (nr) database with the BLASTP algorithm.

**Total RNA preparation**

Total RNA of tissues or cells was extracted using the RNeasy Mini kit (Qiagen). Meanwhile, the RNase-Free DNase set (Qiagen) was used according to the manufacturer’s protocol to prevent DNA contamination.

**RT (reverse transcription)—PCR and qRT-PCR (quantitative real-time PCR)**

For each RNA sample, 2 μg was reverse-transcribed using a first-strand cDNA synthesis kit (Invitrogen). Then, the first-strand cDNA was used to amplify genes of interest with gene-specific primers listed in Supplementary Table S1 (at http://www.BiochemJ.org/bj/443/bj4430779add.htm). The number of PCR cycles was optimized for each gene to ensure linear amplification. To examine developmental expression of the Mageb18 gene, testes were harvested from mice on 1, 7, 14, 21, 28, 35, 42, 49 and 56 days after birth. RNA isolation and RT–PCR were performed as described above. The impact of DNA methylation and histone acetylation on the expression of Mageb18 was quantified using qRT-PCR with SYBR Green I (Invitrogen) and gene-specific primers showed in Supplementary Table S1. RNA levels were normalized using β-actin.

**Construction of MAGEB18 expression vector and transfection**

To overexpress the MAGEB18 protein, the Mageb18 gene was amplified directly from mouse testis cDNA with the primers indicated in Supplementary Table S1. After sequencing verification, the gene was subcloned into the pcMV-HA expression vector with SalI and KpnI sites. Finally, the recombinant plasmid pcMV-HA-Mageb18 was transfected into COS7 or HEK-293 cells with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol.

**Protein extracts and Western blotting**

Total protein extracts of different cell lines and mouse normal tissues were prepared using RIPA buffer (Beyotime). Then, 30 μg of total proteins for each sample were separated by SDS/PAGE (12% gels) and humectate-blotted onto Immobilon-P transfer membranes (Millipore). After blocking, the membrane was probed with primary antibodies against the proteins of interest. Finally, the proteins were further detected using the HRP (horseradish peroxidase)-conjugated secondary antibody and chemiluminescence HRP substrate kit (Millipore). The primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit anti-Bax (P-19) and anti-p21 (P-16) primary goat anti-MAGEB18 (I-12) polyclonal antibody, mouse anti-p53 (clone 2Q366) and anti-(caspase 3) (clone G-5) monoclonal antibodies, and rabbit ant
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for 1 h at room temperature, the cells were incubated overnight at 4°C with goat anti-MAGEB18 (I-12) antibody or mouse anti-HA antibody (1B10). After three washes with PBS, FITC-labelled rabbit anti-(goat IgG) or goat anti-(mouse IgG) (Sigma) secondary antibody was applied for 45 min at room temperature. Nuclei were counterstained with 1 mg/ml DAPI (4′,6-diamidino-2-phenylindole) for 5 min. Finally, the cells were mounted in 80% glycerol and analysed with a Leica TCP SP5 laser-scanning microscope (Leica) at ×63 magnification.

Immunohistochemical staining

Formalin-fixed paraffin-embedded mouse testis sections were processed for immunohistochemical staining. Briefly, slides were deparaffinized in xylene, and rehydrated in a graded alcohol series. Then, the endogenous peroxidase activity was blocked with 1.5% hydrogen peroxide and antigen retrieval was processed by heating the slides in 10 mM sodium citrate buffer (pH 6.0) for 20 min at 95°C. Non-specific background staining was minimized by pre-incubating the sections with 0.3% BSA for 1 h at room temperature. Then, slides were washed in PBS for 10 min and incubated with the goat anti-MAGEB18 (I-12) antibody overnight at 4°C. After washing with PBS, the slides were incubated with HRP-conjugated rabbit anti-goat IgG (Tianjin Sungene) following the manufacturer’s instructions. Briefly, the siRNA-transfected B16-F0 cells were trypsinized after 72 h of transfection. Cells were washed twice with ice-cold PBS and then resuspended in binding buffer from the kit. APC–annexin V and 7-AAD (7-aminoactinomycin D) staining kit (Beyotime). Finally, the slides were counterstained with haematoxylin and then mounted with Permount Mounting Medium (Fisher Scientific). Mouse isotype serum was used as a negative control.

Knockdown of Mageb18 by siRNA (small interfering RNA)
siRNAs targeting Mageb18 (siMageb18) were designed and synthesized by GenPharm. A non-related sequence provided by GenPharm was used as an siRNA negative control (siNC). The sequences of all siRNAs are shown in Supplementary Table S1. The siRNAs were transfected into B16-F0 cells with 75% confluency in six-well culture plates using Lipofectamine™ 2000 (Invitrogen). At 48 h after transfection, cells were lysed and Western blotting was used to analyse the knockdown efficiency.

Detection of cell viability and proliferation capacity in vitro

To analyse the effects of Mageb18 on cell viability and proliferation capacity, cell growth curves and colony formation assays without soft agar [17] were used. For the cell growth curve assay, siRNA-treated B16-F0 cells were seeded in triplicate in 24-well plates at 5 × 10³ cells/well. Cells were trypsinized, stained with Trypan Blue and counted over a 7-day period starting the day after staining. Then, the growth curves were drawn according to the number of live cells. For the colony formation assay, B16-F0 cells transfected with siRNA were seeded into six-well plates (1 × 10⁴ cells/well). After inoculation for 14 days, the colonies were stained with Crystal Violet solution (Allied Chemical Corporation) for 15 min. Then, the number of colonies over 50 cells was counted. The colony formation ratio was calculated using the equation:

\[
\text{colony formation ratio} = \frac{\text{number of colonies}}{\text{number of seeded cells}} \times 100
\]

In vivo tumorigenesis assay

C57BL/6J mice (6–7 weeks old) were randomly assigned to one of two groups (ten mice per group). B16-F0 cells transfected with siMageb18 or siNC were trypsinized and resuspended at a final concentration of 2 × 10⁶ cells/ml in PBS. Then, 100 μl of cells were injected subcutaneously into the right flank of the mice. Tumour growth was monitored and recorded by measuring tumour length and width daily for 16 days, and tumour volume was calculated using the formula:

\[
0.5 \times \text{length} \times \text{width}^2
\]

Apoptosis analysis

Cell apoptosis was measured using the APC (allophycocyanin)– annexin V and 7-AAD (7-aminoactinomycin D) staining kit (Tianjin Sungene) following the manufacturer’s instructions. Briefly, the siRNA-transfected B16-F0 cells were trypsinized after 72 h of transfection. Cells were washed twice with ice-cold PBS and then resuspended in binding buffer from the kit. APC–annexin V and 7-AAD were added into the culture tube. Finally, flow cytometry analysis was performed within 1 h using FACS Calibur (BD Biosciences).

Statistical analysis

Data are expressed as means ± S.E.M. Student’s t test was used to evaluate the significance of differences between sample means obtained from three independent experiments. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Mageb18 is a type I MAGE gene conserved in higher mammals

While screening the EST database in GenBank®, we identified three ESTs (GenBank® accession numbers BY725125.1, BB327259.2 and BB645562.1) within a single unigene cluster, Mm327999. These three ESTs were cloned from the cDNA libraries of cerebellum or adipose tissues. We then assembled these ESTs into a preliminary consensus sequence, and this sequence was used to identify IMAGE consortium clones containing large inserts. As shown in Figure 1, the complete sequence of these clones revealed a 2160 bp mRNA which shared 100% homology with mouse Mageb18 (GenBank® accession number NM_173783.3). It is well known that many MAGE genes are capable of using different exons to produce multiple isoforms [18]; however, the ESTs present in the unigene cluster did not suggest the existence of alternatively spliced isoforms of mouse Mageb18.

The largest ORF (open reading frame) of mouse Mageb18 is 984 bp, which is flanked by a 404 bp 5′-UTR (untranslated region), a 772 bp 3′-UTR and a poly-A signal (A₂¹₃TAATA) (Figure 1). The 984 bp ORF encodes a predicted protein that contains 327 amino acids with an estimated molecular mass of 37203.31 Da and a theoretical pI of 6.43. The MAGEB18 protein contained a MAGE N-terminal domain and a conserved MHD (MAGE homology domain), which are located in Arg¹–Asp⁶ and Ile⁹¹–Ala³⁹⁰ respectively. TargetP 1.1 predicted that the protein carried no subcellular localization sequences. However, eight potential phosphorylation sites are predicted in this protein, including six protein kinase CK2 phosphorylation sites (Ser⁶⁶, Thr-Ser-Asp, Ser³⁸-Ser-Asp-Asp, Thr¹¹³-Lys-Ala-Asp, Ser¹⁶⁸).

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Glut-Thr-Glu, Thr77-Thr-Pro-Asp and Thr284-Leu-Tyr-Glu, a cAMP- and cGMP-dependent protein kinase phosphorylation site (Lys134-Arg-Ala-Ser) and a protein kinase C phosphorylation site (Thr124-Ser-Lys). A potential N-myristoylation site is also present at Gly210-Val-Tyr-Ala-Asn-Arg. Interestingly, all of the post-translational modification sites are located in the MHD, with the exception of two phosphorylation sites, Ser66-Thr-Ser-Asp and Ser76-Ser-Asp-Asp.

Previous studies have indicated that the type I MAGE genes are located on the X chromosome and form several gene clusters [1,19]. We have shown that mouse Mageb18 is also located on chromosome Xc2-C3, which is closely linked to another seven MAGEB genes (Figures 2A and 2B). Phylogenetic analysis of the mouse MAGE genes on chromosome X indicates that Mageb18 is formed in the late stages of the MAGEB subfamily (Figure 2C). These results collectively suggested that mouse Mageb18 belongs to the type I MAGE genes.

We also used the mouse MAGEB18 protein (GenBank® accession number NP_776144.1) to screen the non-redundant protein sequence database. As shown in Supplementary Figure S1 (at http://www.BiochemJ.org/bj/443/bj4430779add.htm), the mouse MAGEB18 protein is located on chromosome Xc2-C3, which is closely linked to another seven MAGEB genes (Figures 2A and 2B). Phylogenetic analysis of the mouse MAGE genes on chromosome X indicates that Mageb18 is formed in the late stages of the MAGEB subfamily (Figure 2C). These results collectively suggested that mouse Mageb18 belongs to the type I MAGE genes.

The expression of Mageb18 is ubiquitous in normal mouse tissues

Previous studies have presumed that the expression of type I MAGE genes is testis-specific [1,19]. However, our preliminary results from EST data have shown that Mageb18 can also be cloned from non-testis cDNA libraries. As shown in Figure 3(A), RT–PCR analysis with total RNA from normal mouse tissues indicated that Mageb18 is indeed expressed in stomach, large intestine, small intestine, spleen, lymph node, bone marrow lymphocytes and blood T-lymphocytes, as well as testis. Moreover, the expression level in lymphocytes from bone marrow and blood is higher than that in other tissues. However, no expression was observed in the brain, heart, lung, liver or kidney. The expression patterns of testis-specific Mageb1 and ubiquitous Magee1 control genes were consistent with previous results [5,20].

Expression of Mageb18 in testis is age-dependent

Previous studies have shown that the cancer/testis MAGE genes expressed in testis play important roles in reproduction and development [9,21]. To analyse further the expression of Mageb18 number XP_003416101.1, rabbit (GenBank® accession number XP_002720034.1), cat (GenBank® accession number NP_001009368.1), hamster (GenBank® accession number EGV98014.1). Sequence alignment indicated that the MHD of MAGEB18 proteins among these mammals is highly conserved, whereas the N- and C-terminal regions are more labile. To date, no orthologous predicted protein sequences have been identified in any other species, suggesting that Mageb18 is a type I MAGE gene conserved in higher mammals.

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Figure 2 The chromosome location and phylogenetic analysis of the mouse Mageb18 gene
(A) Ideogram of mouse chromosome X. The brackets indicate the relative location of the MAGE gene clusters. (B) Arrangement of the mouse Mageb cluster on chromosome X. The gene clusters were derived from the assembled genomes of mouse (NCBI build 37.2). The Mageb cluster is split into two separate loci on chromosome XC1-C3 from 83.5 to 90.0 Mb. The position of each gene is represented by a vertical bar. The direction of transcription is indicated by the arrows. Note that the black arrowhead represents the functional genes, whereas the white arrowhead indicates the pseudogenes. (C) The phylogenetic tree of mouse MAGE genes in chromosome X. The tree was constructed by the neighbour-joining method based on the proportional difference (p-distance) of aligned amino acid sites of the MHD. In total, 1000 bootstrap replicates were used to test the reliability of each branch.

in testis, age-dependent expression was analysed in 1–56-day-old mice. As shown in Figure 3(B), Mageb18 expression was detected from the first day of birth, and found to increase steadily in the first 3 weeks of life. Mageb18 reached full expression between 14 and 21 days and had a stable expression level between 21 and 56 days. Further Western blotting analysis using protein extracts also yielded similar results (Supplementary Figure S2 at http://www.BiochemJ.org/bj/443/bj4430779add.htm). The expression of Mageb18 in testis was throughout this age range and reached a maximum expression in early puberty, indicating a role for Mageb18 in both testis development and spermatogenesis.

DNA demethylation and histone acetylation mediate the reactivation of Mageb18 in mouse cell lines
Several studies have shown that promoter methylation and histone acetylation regulate the expression of MAGE genes [22,23]. To investigate whether these epigenetic mechanisms also mediate the expression of Mageb18, we analysed its expression in eight mouse-derived cell lines using RT–PCR. As shown in Figure 3(C), Mageb18 mRNA expression was detected in a broad range of cell lines, including B16-F0 (melanoma), 4T1 (breast cancer), L929 (fibroblasts), NIH 3T3 (embryonic fibroblasts), MM45T.Li (liver cancer) and RAW264.7 (macrophages). However, the hepatocellular carcinoma cell line H22 and glioma cell line C6 showed no Mageb18 mRNA expression.

Next, qRT–PCR was used to clarify whether both DNA methylation and histone deacetylation are involved in transcriptional repression of Mageb18 in H22 and C6 cell lines. As shown in Figure 3(D), H22 and C6 cells treated with TSA alone had 7.7- and 7.9-fold higher transcript levels of Mageb18 than the untreated basal levels respectively. Treatment of the cell lines with 5-aza-Cdr alone had a small influence on Mageb18 expression, which increased only 3.1-fold in H22 and 2.2-fold in C6 cells compared with the untreated control. However, 5-aza-Cdr was able to synergistically enhance TSA-mediated Mageb18 transcription. The expression level could reach 18.8- and 17.5-fold higher levels than the untreated basal levels in H22 and C6 cell lines respectively. These results indicated that 5-aza-Cdr and TSA indeed were able to reactivate the expression of Mageb18 in Mageb18-negative H22 and C6 cells, suggesting that DNA demethylation and histone acetylation certainly play important roles in regulating Mageb18 expression.

The mouse Mageb18 gene encodes a 46 kDa protein
To characterize the MAGEB18 protein, HEK-293 cells were transiently transfected with a plasmid encoding HA-tagged
MAGEB18 protein. Western blotting analysis indicated that antibodies against the HA tag or MAGEB18 both detected a unique protein band at approximately 46 kDa (Figure 4A). The protein extracts from brain, heart, lung, liver, stomach, large intestine, small intestine, spleen, lymphoid node, bone marrow lymphocyte, blood T-lymphocyte and testis were immunodetected using an anti-MAGEB18 antibody. As shown in Figure 4(B), no specific signal was observed in the brain, heart, lung, liver and kidney tissues. However, a strong signal was detected at approximately 46 kDa in the stomach, large intestine, small intestine, spleen, lymphoid node, bone marrow lymphocyte, blood T-lymphocyte and testis. These results are consistent with the Mageb18 mRNA expression profile shown in Figure 3(A). Another band at approximately 26 kDa can also be detected in all MAGEB18-positive tissues, but not in MAGEB18-negative tissues. The MAGEB18 protein in mouse-derived cell lines exists as a unique band at approximately 46 kDa (Figure 4C). These results indicated that the endogenous MAGEB18 protein in tissues and cell lines may undergo different post-translational modifications and result in the formation of products with different sizes.

MAGEB18 protein is predominantly localized in the cytoplasm

Because MAGEB18 contained no potential location signal sequence, the localization of full-length MAGEB18 was analysed. To do this, full-length Mageb18 was tagged with an HA epitope at the N-terminus. When HA–MAGEB18 was overexpressed in COS7 cells and immunostained with an anti-HA antibody, the results showed that the MAGEB18 fusion protein was mainly localized in the cytoplasm (Figure 5A). The COS7 cells with mock-transfection showed no staining with the anti-HA antibody (Supplementary Figure S3A at http://www.BiochemJ.org/bj/443/bj4430779add.htm). Next, the MAGEB18-positive B16-F0 cell line was stained with an anti-MAGEB18 antibody, which indicated that the endogenous MAGEB18 protein was also predominantly localized in the cytoplasm (Figure 5B). Moreover, the signal intensity was reduced greatly by siRNA targeting the Mageb18 gene (Supplementary Figure S3B).

Although the HA–MAGEB18 protein overexpressed in COS7 cells and the endogenous MAGEB18 protein in B16-F0 cells are mainly localized in the cytoplasm, the nuclear localization can also be observed in both of the cells. Finally, the mouse testis sections were used to further analyse MAGEB18 expression with

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immunohistochemical staining. As shown in Figure 5(C), by using an antibody against Mageb18, a particularly intense staining signal was observed in spermatogonia attached to the basement membrane of the seminiferous tubules. In addition, Mageb18 was also observed in primary and secondary spermatocytes, but less so in spermatids. With regard to the subcellular distribution, immunoreactivity of Mageb18 was seen mainly in the cytoplasm of spermatogonia. No specific staining of spermatogonia was observed when the primary antibodies were replaced by isotype control serum. Collectively these results demonstrate that the Mageb18 protein is a cytoplasmic protein which also localizes in the nuclei.

**Knockdown of Mageb18 decreased the growth of B16-F0 cells**

To characterize further the function of Mageb18 in regulating cancer cell malignant phenotypes, we used siRNA technology to knock down endogenous Mageb18 in B16-F0 cells. As shown in Figure 6(A), the No.1 siRNA of Mageb18 (siMageb18 1#) can significantly reduce the expression of Mageb18, as confirmed by Western blotting. Therefore this siRNA was used to perform all of the following functional experiments. Next, we analysed the impact of siMageb18 on the growth of B16-F0 cells in vitro. The results indicated that the growth of the cells transfected with siMageb18 was significantly inhibited in comparison with the siNC-treated cells (Figure 6B). To confirm the inhibitory effect of siMageb18 on cell proliferation, we carried out colony formation assays. As shown in Figures 6(C) and 6(D), cells transfected with siMageb18 dramatically inhibited the colony formation ratio compared with the siNC-transfected cells. These results suggested that knockdown of Mageb18 suppressed cell proliferation in vitro.

To investigate whether the decreased viability resulting from Mageb18 knockdown in cell culture can translate into similar effects on B16-F0 cells in vivo, C57BL/6J mice were injected subcutaneously with B16-F0 cells transfected with siNC or siMageb18. As shown in Figures 6(E) and 6(F), knockdown of Mageb18 in B16-F0 cells with siMageb18 resulted in a significant delay in tumour growth, with smaller sizes than in cells treated with siNC. A similar growth-inhibitory effect was also obtained in breast cancer 4T1 cells after suppressing Mageb18 (Supplementary Figure S4 at http://www.BiochemJ.org/bj/443/bj4430779add.htm). Collectively these results demonstrate that knockdown of Mageb18 can inhibit cell proliferation both in vitro and in vivo.

**Knockdown of Mageb18 induced apoptosis in B16-F0 cells**

To investigate whether the inhibition of proliferation by siMageb18 was due to the promotion of apoptosis, we performed APC–annexin V/7-AAD staining followed by FACS analysis. As shown in Figure 7(A), the subpopulations of APC–annexin V−/7-AAD− represented viable cells. The APC–annexin V+/7-AAD− and APC–annexin V+/7-AAD+ subpopulations represented cells under early apoptosis and late apoptosis respectively. Meanwhile, the APC–annexin V−/7-AAD+ subpopulation represented necrotic cells. Knockdown of Mageb18 significantly increased the percentage of total apoptotic cells, which were counted as annexin V+ (Figure 7B). By further analysing apoptosis...
process.

That knockdown of MAGEB18 induced apoptosis in B16-F0 cells, increased in MAGEB18-knockdown cells. These results suggest the level of another pro-apoptotic protein, caspase 3, was found to be also increased the expression of Bax protein. Additionally, the Bax protein. The results showed that knockdown of MAGEB18 TP53 and its target gene p21. Because TP53 is a key modulator knockdown of MAGEB18 indeed increased the protein levels of genes using Western blotting. As shown in Figures 7(C) and 7(D), of TP53, we examined the protein levels of TP53, p21, Bax and caspase 3.

It has been reported that MAGE–RING protein complexes comprise a family of E3 ubiquitin ligases and promote the degradation of TP53 (tumour protein 53) via the ubiquitin–proteasome pathway [24]. To elucidate which the induction of apoptosis by siMageb18 was related to enhancing the stability of TP53, we examined the protein levels of TP53 and its target genes using Western blotting. As shown in Figures 7(C) and 7(D), knockdown of MAGEB18 indeed increased the protein levels of TP53 and its target gene p21. Because TP53 is a key modulator of the pro-apoptotic protein Bax, we next analysed the level of Bax protein. The results showed that knockdown of MAGEB18 also increased the expression of Bax protein. Additionally, the level of another pro-apoptotic protein, caspase 3, was found to be increased in MAGEB18-knockdown cells. These results suggest that knockdown of MAGEB18 induced apoptosis in B16-F0 cells, and that TP53 played an important role in this apoptosis-inducing process.

**DISCUSSION**

The MAGE multigene family is composed of ten subfamilies that can be categorized into two types [1,19]. The A and B subgroups of type I MAGE genes are conserved among mammals, and they encode epitopes for HLA in cancer cells. Type II genes are relatively ancient and have been found in chicken [25], fruitfly [26] and zebrafish [27]. The type I MAGE proteins have attracted considerable attention because some studies have indicated that they are shared tumour-specific antigens which are expressed by many different types of tumours, but not in normal tissues, except the testis or ovary [2]. However, the MAGE genes whose expression profile in normal human tissues have been carefully characterized to date only include MAGEA1 [28], MAGEA10 [29], MAGEB2 [5], MAGEB5, MAGEB6, MAGEC2 and MAGEC3 [4]. Therefore much more attention must be paid to studying the expression and function of other type I MAGE genes because of their important status in cancer immunotherapy.

According to the chromosome location and phylogenetic analysis, Mageb18 is a type I MAGE gene conserved among higher mammals (Figure 2 and Supplementary Figure S1), suggesting that its expression in normal tissues should be testis-specific. However, we first identified three ESTs of Mageb18 from non-testis CDNA libraries. Moreover, further expression analysis using RT–PCR and Western blotting demonstrated that, besides testis, Mageb18 is indeed expressed in stomach, small intestine, small intestine, spleen, lymphoid node, bone marrow lymphocytes and blood T-lymphocytes (Figures 3A and 4B). Interestingly, these tissues belong to two independent digestive and immune systems. Because the various tissues of the digestive system are the locations with the maximum level of pathogens in the body, the expression of Mageb18 in digestion- and immune-related tissues suggests its important role in infection, inflammation and immunity. Such functions have been reported for MAGEA1 in human skin, which has shown that the expression of Mageb18 was activated during the inflammatory phase of wound healing [30].

Although the specific mechanism of regulating Mageb18 tissue-specific expression is unclear, previous studies have shown that promoter demethylation and histone acetylation mediate expression of MAGEA1, MAGEA2, MAGEA3, MAGEA6 and MAGEA12 in human cancer cells [22,31]. In the present study, we found that the expression of Mageb18 was also regulated by these epigenetic mechanisms (Figure 3D). It is well known that most demethylation processes occur in the CpG islands located in the promoter region. However, no CpG islands were found in the promoter region for most of the MAGE genes (including Mageb18), suggesting that the non-CpG sites in the promoter region and/or CpG sites in non-promoter region may play important roles in regulating the MAGE gene expression [32,33].

Bioinformatics predicted that the Mageb18 gene encoded a protein with a molecular mass of 37 kDa. However, Western blotting analysis indicated that the molecular mass of endogenous Mageb18 is 46 kDa (Figure 4), which is slightly larger than the predicted size, but consistent with that of other MAGE proteins of the same length, such as Mageb18 [34], MAGEA3 [35] and Mageb2 [5]. Furthermore, another band was observed at approximately 26 kDa in mouse normal tissues that expressed Mageb18, but this was not seen in mouse-derived cell lines (Figure 4B). These results suggest that the endogenous Mageb18 protein certainly undergoes some post-translational modification, such as phosphorylation and protease cleavage. A previous study has also reported that a cleaved C-terminal fragment with pro-apoptotic activity was generated from full-length Magea4 after genotoxic stress in human cells [36]. Sequence analysis in the present study also indicated that Mageb18 protein indeed possesses several post-translational modification sites, and most of these sites are located in the MHD of Mageb18 protein (Figure 1). A recent study on the crystal structures of MAGE–RING complexes has revealed that the MHD of MAGE proteins form two conserved winged-helix motifs [24]. Therefore the modifications in the MHD of Mageb18 protein may play important roles in regulating the stability of its three-dimensional structure, which is vital to mediate different protein–protein interactions under various physiological conditions.

The subcellular localization of a protein is very important for its physiological activity, so we further investigated the
subcellular localization of MAGEB18. Our immunostaining results from cells and tissues indicated that the MAGEB18 protein is predominantly localized in the cytoplasm, but can also be seen in the nuclei (Figure 5). Because MAGEB18 contains no potential nuclear localization signal sequence, the nuclear localization of MAGEB18 suggests that it may interact with some nuclear proteins, such as TP53 [37]. Furthermore, many studies have also shown that it is common for MAGE proteins in cells to function as adaptors and mediate protein–protein interactions in an evolutionarily conserved mode with other partners, such as the EID (E1A-like inhibitor of differentiation) families [38], RING E3 ubiquitin ligases [24] and the KAP1 scaffolding protein [39]. Therefore identification of small-molecule compounds or peptides that inhibit protein–protein interactions between MAGEs and their partners will be a potential strategy for development of novel chemo-therapeutic agents for treatment of advanced cancers [40].

The functions of MAGE gene products have not so far been well characterized. However, previous reports have demonstrated that MAGE genes were expressed in undifferentiated proliferating cells. For example, Magea1 and -A4 were expressed in testicular cells in the proliferative phase (spermatogonia), but not in the differentiation phase (spermatids) during spermatogenesis [41]. The mouse homologues of human MAGE genes are also expressed in mouse blastocysts and embryonic stem cells [42]. These findings suggest that the products of MAGE genes certainly play important roles in the proliferating phase of cells. The results of the present study indicate that MAGEB18 was also predominantly expressed in proliferative spermatogonia, and primary and secondary spermatocytes (Figure 5C). Moreover, we found that MAGEB18 indeed regulates cell proliferation of B16-F0 melanoma cells (Figure 6) and 4T1 breast cancer cells (Supplementary Figure S4) both in vitro and in vivo. Knockdown of MAGEB18 inhibited the growth of B16-F0 cells and induced apoptosis (Figures 6 and 7). Although the specific mechanisms are currently not completely clear, loss of MAGEB18 has been demonstrated to promote the expression of TP53 and finally activate its target genes, such as p21 and Bax (Figure 7C), demonstrating to promote the expression of TP53 and finally activate its target genes, such as p21 and Bax (Figure 7C), suggesting that TP53 may be an important mediator mediating these processes. A recent study has indeed shown that loss of MAGEA3 led to apoptosis mediated by p53-dependent activation of pro-apoptotic Bax expression in myeloma cells [43].

In summary, the present study has shed light on the mysterious expression and functions of MAGEB18 proteins. We have shown that this type I MAGE gene is expressed ubiquitously in normal tissues and regulates proliferation and apoptosis of cancer cells. Therefore the results suggest a need to study further the expression pattern of other type I MAGE genes in normal tissues prior to using them to develop more effective and safer cancer vaccines.

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SUPPLEMENTARY ONLINE DATA

The mouse Mageb18 gene encodes a ubiquitously expressed type I MAGE protein and regulates cell proliferation and apoptosis in melanoma B16-F0 cells

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Figure S1 Amino acid sequence alignment of MAGEB18 orthologues from mammals

MAGEB18 has been identified in mouse (NP_776144.1), rat (XP_228594.4), dog (XP_548906.1), giant panda (EFB13851.1), pig (XP_003135033.1), cattle (XP_002700367.1), human (NP_775870.1), chimpanzee (XP_528911.1), gibbon (XP_003262121.1), horse (XP_001493441.2), elephant (XP_003416101.1), rabbit (XP_002720034.1), cat (NP_001000368.1) and hamster (EGV98014). Dashes are inserted to optimize the alignment, and conserved residues are shaded.

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Figure S2  Age-dependent expression of MAGEB18 protein in mouse testis

Protein extracts from 1–56-day-old mouse testes were used, and MAGEB18 was immunodetected using an anti-MAGEB18 antibody. β-Actin served as an internal control. The molecular mass in kDa is indicated on the left-hand side. ACTB, β-actin.

Figure S3  The specificity of anti-HA and anti-MAGEB18 antibodies used in indirect immunofluorescent staining

(A) COS7 cells with mock transfection were stained with an anti-HA antibody (green), and nuclei were defined by DAPI staining (blue). Scale bar = 20 μm. (B) B16-F0 cells were transfected with siMageb18. The cells were stained with an anti-MAGEB18 antibody (green) 48 h after transfection. Nuclei were defined by DAPI staining (blue). Scale bar = 20 μm.

Figure S4  Knockdown of MAGEB18 decreased the growth of 4T1 cells both in vitro and in vivo

(A) Western blotting was used to evaluate the knockdown efficiency of siRNAs targeting the Mageb18 gene. (B) Knockdown of MAGEB18 inhibited cell proliferation. After 24 h of transfection with siMageb18 or siNC, 4T1 cells were seeded in 24-well plates at 5 × 10^3 cells/well. Cells were grown for 1–7 days and the number of live cells was counted every day. (C) Knockdown of MAGEB18 inhibited colony formation. The siRNA-transfected 4T1 cells were seeded into six-well plates at 1 × 10^3 cells/well. After a period of 14 days, the cells were stained with Crystal Violet. Representative images from three separate experiments are shown (top panel). Bottom panel: the number of colonies over 50 cells shown in the top panel was counted and the colony formation ratio was calculated. Results are means ± S.E.M. **P < 0.01. (D) Knockdown of MAGEB18 delayed tumour development in vivo. The siMageb18 or siNC-treated 4T1 cells (2 × 10^5 cells/mouse) were injected subcutaneously into Balb/c mice (ten mice/group) at day 0. Tumour growth was observed and recorded daily. Tumour size over 3 mm × 3 mm was considered positive. Kaplan–Meier curves showing the percentage of mice without tumour from one representative experiment. **P < 0.01. (E) The tumour size (mean ± S.D.) as shown in (D) was calculated. *P < 0.05, **P < 0.01.
**Table S1**  Summary of the primers and siRNAs sequences used in the present study

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