

MAGE-D1 inhibits proliferation, migration and invasion of human breast cancer cells

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Abstract. MAGE-D1, also known as NRAGE or Dlxin-1, is a member of the MAGE family of proteins. It interacts with multiple adaptors and mediates various cellular functions such as regulation of apoptosis, transcription, cell cycle, cell adhesion and angiogenesis. In this study, we evaluated the effect of MAGE-D1 plasmid transfection on the growth, migration and invasion of breast cancer cells. MTT assay and cell counting consistently showed that MAGE-D1 transfection could effectively inhibit the proliferation of breast cancer cells. However, further FACS analyses failed to demonstrate any alterations in cell cycle distribution and apoptosis after MAGE-D1 transfection. *In vitro* scratch wound healing assay exhibited that MAGE-D1 suppressed cell migration. In addition, Boyden chamber invasion assay showed that MAGE-D1 significantly inhibited cell invasion. Furthermore, in an attempt to elucidate the mechanism of MAGE-D1 in suppressing cellular growth and invasion, the protein expressions of p53, p21, E-cadherin, β -catenin, MMP-2 and MMP-9 were assessed. Western blotting showed that MAGE-D1 up-regulated the expression of p53, p21 and E-cadherin, whereas down-regulated β -catenin expression. Taken together, this study suggests that MAGE-D1 play important roles in the regulation of cell proliferation, migration and invasion of breast cancer cells. Enhanced expression of MAGE-D1 by gene transfer could reverse the malignant phenotypes of breast cancer cells. MAGE-D1 may be a potential therapeutic target for breast cancer.

Introduction

Breast carcinoma is the most common malignancy in women and represents the second leading cause of cancer death

among women (just after lung cancer). Germ line mutations in the breast cancer susceptibility gene BRCA2 confer susceptibility to familial early-onset breast and ovarian cancers (1,2). However, BRCA2 is rarely mutated in sporadic breast and ovarian cancers (3). It is possible that distinct mechanisms targeting the BRCA2 pathways operate in sporadic cases. To identify molecules involved in BRCA2 functions, yeast two hybrid screening was carried out and MAGE-D1 (MAGE for melanoma antigen) was identified as a BRCA2 binding protein (4). The authors showed that BRCA2 suppressed cell proliferation via stabilizing MAGE-D1. MAGE-D1 acted as a downstream target of BRCA2 and mediated the growth-suppressing function of BRCA2, which indicates that disruption of the MAGE-D1 function by suppression of expression, mutation or deletion should be functionally equivalent to BRCA2 mutations in disrupting BRCA2 function. Consistent with this hypothesis is their finding that, as compared with MAGE-D1 expression in untransformed mammary cell lines, the expression level of MAGE-D1 was reduced in 6 of the 16 breast carcinoma cell lines. This suggests that suppression of MAGE-D1 expression may be involved in the development of a subset of sporadic breast cancers. Therefore, we were curious to know whether MAGE-D1 up-regulation could reverse the malignant phenotypes of breast carcinoma cells.

MAGE-D1, also known as NRAGE or Dlxin-1, is a member of the MAGE family of proteins (5,6). Over 32 MAGE proteins have been identified in humans, but the normal physiological function of this family remains unclear (7). Members of the hMAGE-A, hMAGE-B and hMAGE-C subfamilies are only expressed in male germ cells or placenta, but completely silent in most normal tissues (5,8). However, many members of the MAGE family are expressed in various tumor cells and thus are of particular interest for antitumor immunotherapy (7). Members of the hMAGE-D, hMAGE-E, hMAGE-F, hMAGE-G and hMAGE-H subfamilies are expressed in many normal tissues at various levels (5,9,10). Noteworthy, mMAGE-D1 expressed the most in brain and ovary among murine tissues (10), indicating that MAGE-D1 may play important physiological roles in female reproductive organs. Previous studies indicate that MAGE-D1 is a regulator of apoptosis, transcription, cell cycle progression, cell adhesion and angiogenesis (5,6,11,12). MAGE-D1 interacted with Dlx/Msx homeodomain proteins and was required for Dlx5-dependent transcription (13). It was reported that MAGE-D1 cooperated with Necdin, another MAGE family protein, to

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regulate the function of Msx homeodomain proteins in cellular differentiation (14). MAGE-D1 also interacts with several apoptosis regulators such as p75 neurotrophin receptor, axon guidance receptor UNC5H, Ror receptor kinases, inhibitors of apoptosis proteins (IAP), ubiquitin ligase praja1, and anti-apoptotic factor Che-1 (15-20). MAGE-D1 has been reported to inhibit adhesion of osteosarcoma U2OS, and suppress metastasis of melanoma and pancreatic cancer *in vitro* and *in vivo*, which indicates that human MAGE-D1 may be involved in cell adhesion and invasion (11,21). Subsequent study demonstrated that overexpression of MAGE-D1 inhibited the angiogenesis *in vitro* and *in vivo* (12).

However, the effects of MAGE-D1 on breast cancer cells remain to be elucidated. In this study, we investigated the effectiveness of MAGE-D1 transfection on the growth, migration and invasion of breast cancer cells for the first time. Our results demonstrated that human MAGE-D1 transfection could partly reverse the malignant phenotypes of breast carcinoma cells.

Materials and methods

Cell culture. The human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM supplemented with 10% FBS (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (North China Pharmaceutical Factory), in a humidified atmosphere of 5% CO₂ at 37°C.

Plasmid construction and cell transient transfection. The construction of human wild-type MAGE-D1 expression vector has been described previously (4). The construct pCDEF3-MAGE-D1 was kindly provided by Dr Qingshen Gao (Evanston Northwestern Healthcare Research Institute, USA). Transient transfection was performed using the FuGENE HD (Roche Applied Science, USA) as described by the manufacturer. Empty vector pCDEF3 was transfected as a control. MCF-7 and MDA-MB-231 cells transfected with pCDEF3-MAGE-D1 or pCDEF3 were named as MAGE-D1/MCF-7 and MAGE-D1/MDA-MB-231, or vector/MCF-7 and vector/MDA-MB-231, respectively.

Cell growth assay. The effect of MAGE-D1 on proliferation was demonstrated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay as described previously (22). In brief, after 6 h of transient transfection, cells were seeded into 96-well plates at 1000 cells/well for MCF-7 or 800 cells/well for MDA-MB-231 cells. Five wells were assigned to each experimental treatment. After transfection for 24, 48 and 72 h, 20 μ l of MTT reagent (5 mg/ml; Sigma) was added to each well and incubated for 4 h. Then, the reaction was stopped by removal of MTT, and 150 μ l DMSO was added into each well in order to dissolve the formazan crystals. Absorbance at 570 nm was recorded using a 96-well microplate reader (Bio-Rad, Hercules, CA, USA).

The effect of MAGE-D1 on proliferation was also assessed by cell counting using a haemocytometer after 1, 2, 3 and 4 days of transfection. All measurements were done in triplicate.

Western blot analysis. After 24 or 48 h of transfection, cells were rinsed twice with ice-cold PBS and lysed in lysis buffer [60 mmol/l Tris-HCl (pH 6.8), 2% SDS, 100 mmol/l DTT, 50 mmol/l NaF, 1 mmol/l Na₃VO₄] (22). Protein concentration was determined by the Bio-Rad Protein assay kit (Bio-Rad). Aliquots of whole-cell lysate (50 μ g) were resolved on SDS-PAGE gels, and transferred electrophoretically to nitrocellulose membranes (Bio-Rad). Blots were probed with primary antibodies against MAGE-D1 (kindly provided by Dr Qingshen Gao), E-cadherin, β -catenin, p53, p21, MMP-2, MMP-9 or actin. All of these primary antibodies except MAGE-D1 were from Santa Cruz Biotechnology Inc. After washing, blots were incubated with a 1:5000 dilution of horseradish peroxidase-linked secondary antibody (Promega). Antigen-antibody complexes were detected by using Enhanced Chemiluminescence reagent according to the manufacturer's instruction (Amersham Pharmacia Biotech, Buckinghamshire, UK).

In vitro scratch wound healing assay. After 24 h of transient transfection, cells were seeded into 6-well plates and grown to confluence. Cell migration was measured by the *in vitro* scratch wound healing assay (23). The confluent cell monolayer was scraped with a sterile pipette tip and wounded monolayer was then washed three times with PBS to remove cell debris. Immediately after wounding and at the end of the experiment, wounds were photographed. The migration ability of the cells was evaluated by measuring the width of the wounds. The migration distances of the cells were measured by the difference of the width of wounds at 0 and 24 h for MCF-7 cells, or at 0 and 6 h for MDA-MB-231 cells.

Transwell invasion assay. A Boyden chamber invasion assay was used to quantify cell invasion (22). Briefly, polycarbonate filters with 8- μ m pores (Millipore) were coated with matrigel (BD Biosciences). After 48 h of transfection, MDA-MB-231 cells (2 \times 10⁵) were seeded into the upper chambers, and the lower compartment was filled with 200 μ l NIH3T3-conditioned medium. Then, MDA-MB-231 cells were incubated for 6 h. Having mechanically removed cells on the upper surface of membranes, the filters were fixed in methanol and stained with hematoxylin and eosin. Cells having migrated onto the lower surface were counted manually under a light microscope, with \times 200 magnification. Each experiment was repeated twice, and five microscopic fields from each filter were counted. Results were expressed as the mean number of cells counted in each field \pm standard deviation.

Flow cytometry. After 24 h of transfection, cells were harvested and fixed in ethanol at 4°C for 24 h. The pellets were resuspended in 100 mg/l RNase and 10 mg/l propidium iodide diluted with phosphate buffer solution (PBS) for 30 min. Apoptosis cell fraction and cell cycle distribution were analyzed by FACScan cytometry (Becton-Dickinson, San Jose, CA, USA) (24).

Statistical analysis. All experiments were repeated at least twice. The data were analyzed with the software package SPSS 12.0. P-value <0.05 were considered significant.

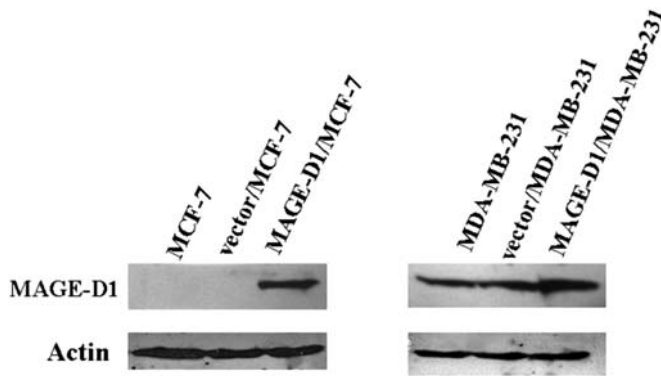


Figure 1. Effect of MAGE-D1 plasmid transfection on MAGE-D1 expression. After 24 h of transfection, the expression levels of MAGE-D1 protein was detected by Western blot analysis in total cell extracts. The actin serves as a loading control. Similar experiments were repeated at least twice.

Results

Effect of MAGE-D1 plasmid transfection on MAGE-D1 expression. The pCDEF3-MAGE-D1 constructs were transfected into breast carcinoma cell lines MCF-7 and MDA-MB-231. The effect of pCDEF3-MAGE-D1 plasmid transfection on the expression of MAGE-D1 protein was evaluated by Western blot analysis using a rabbit anti-human MAGE-D1 polyclonal antibody. After 24 h of transfection, MCF-7 cells transfected with empty vector (denoted as vector/MCF-7) as well as parental cells expressed undetectable level of MAGE-D1 protein, MCF-7 cells transfected with pCDEF3-MAGE-D1 constructs (denoted as MAGE-D1/MCF-7) expressed high level of MAGE-D1 protein (Fig. 1). After 48 h of transfection, MDA-MB-231 cells transfected with pCDEF3-MAGE-D1 constructs (denoted as MAGE-D1/MDA-MB-231) expressed about 2-fold higher level of MAGE-D1 protein than MDA-MB-231 parental cells and cells transfected with empty vector (denoted as vector/MDA-MB-231) (Fig. 1). These results suggest that pCDEF3-MAGE-D1 construct transfection can effectively up-regulate MAGE-D1 expression.

Effect of MAGE-D1 plasmid transfection on the proliferation of breast cancer cells. Two proliferation analyses, MTT and cell counting, were used to assess the proliferative ability of breast cancer cells transfected with pCDEF3-MAGE-D1 plasmid. MTT assay showed that pCDEF3-MAGE-D1 plasmid transfection significantly inhibited the cellular growth after 2 and 3 days of transfection, relative to the empty vector transfected cells ($P < 0.05$) (Fig. 2A). There was no significant difference in the cell growth rate between empty vector transfected cells and parental cells ($P > 0.05$).

Cell counting assay also demonstrated the anti-proliferative effects of pCDEF3-MAGE-D1 plasmid transfection on MDA-MB-231 and MCF-7 cells (Fig. 2B). After 3 days of transfection, growth of MAGE-D1/MCF-7 and MAGE-D1/MDA-MB-231 was inhibited by 27.4 and 60.0%, respectively, relative to the empty vector-transfected cells.

Effect of MAGE-D1 plasmid transfection on cell cycle and cell apoptosis of breast cancer cells. To determine the role of

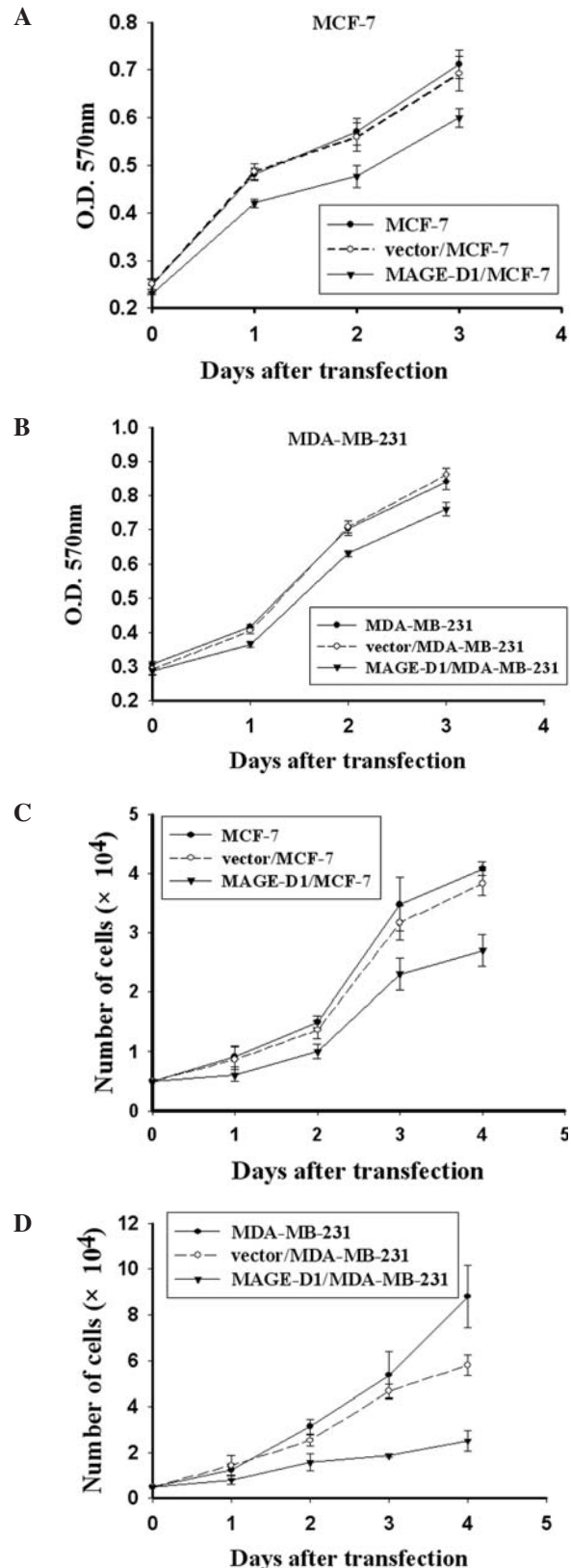


Figure 2. Effect of MAGE-D1 plasmid transfection on the proliferation of human breast cancer cells. (A and B) MTT assay to examine the anti-proliferative effects of MAGE-D1. After 6 h of transient transfection, cells were seeded into 96-well plates at 1000 cells/well for MCF-7 or 800 cells/well for MDA-MB-231 cells. Five wells were assigned to each experimental treatment. Experiments were repeated three times, data are presented as means \pm SD (vertical bars). (C and D) Cell counting to assess the anti-proliferative effects of MAGE-D1. Cells were transfected with MAGE-D1 plasmid. Proliferation was evaluated by cell counting using a haemocytometer after 1, 2, 3 and 4 days of transfection. Experiments were repeated three times, data are presented as means \pm SD (vertical bars).

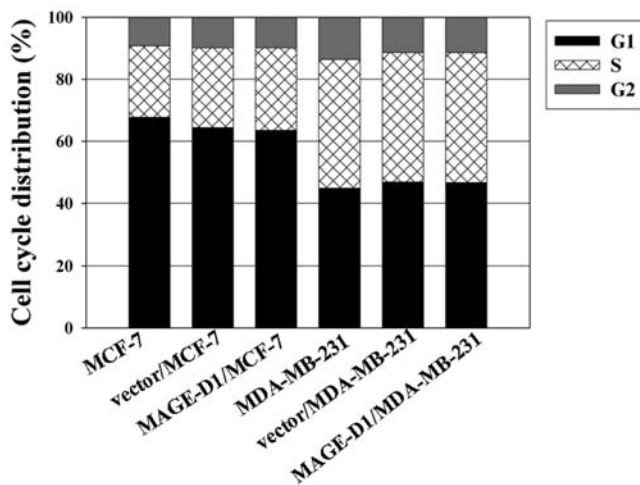


Figure 3. Effect of MAGE-D1 plasmid transfection on cell cycle distribution of breast cancer cells. Flow cytometric assay was used to analyze cell cycle distribution. Data are presented as means of three independent experiments.

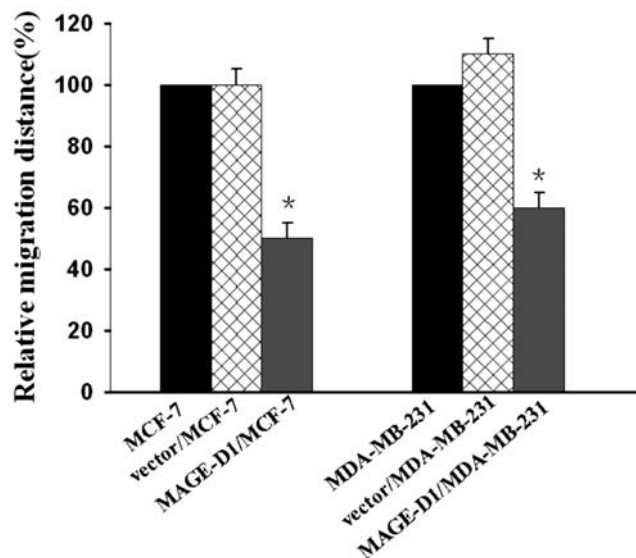


Figure 4. Effect of MAGE-D1 plasmid transfection on migration of breast cancer cells. *In vitro* scratch wound healing assay was used to quantify cell migration. Data are presented as means \pm SD (vertical bars) of three independent experiments. * $P < 0.01$ vs. vector transfected cells.

MAGE-D1 in cell cycle distribution and cell apoptosis, flow cytometric analysis was performed in breast cancer cells. Transfection of MAGE-D1 did not affect cell cycle distribution (Fig. 3) as well as apoptosis (data not shown) in MCF-7 and MDA-MB-231 cells.

Effect of MAGE-D1 plasmid transfection on migration of breast cancer cells. To determine whether MAGE-D1 could affect cell migration, a wound healing assay was carried out in breast cancer cells. After 48 h of transfection, confluent and quiescent monolayers of MAGE-D1/MCF-7, vector/MCF-7, parental MCF-7, MAGE-D1/MDA-MB-231, vector/MDA-MB-231, parental MDA-MB-231 cells were wounded. Fig. 4 showed that MAGE-D1/MCF-7 and MAGE-D1/MDA-MB-231 cells had a much slower wound-healing rate

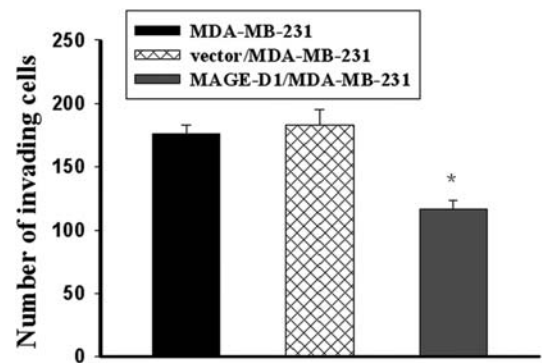


Figure 5. Effect of MAGE-D1 plasmid transfection on invasion of breast cancer cells. A boyden chamber invasion assay was used to quantify cell invasion. After 24 h of transfection, MDA-MB-231 cells (2×10^5) were seeded into the upper chambers, and incubated for 6 h. Then the cells having migrated onto the lower surface were counted manually under a light microscope, with $\times 200$ magnification. Data are presented as the mean number of cells counted in each field \pm SD (vertical bars) of three independent experiments. * $P < 0.01$ vs. vector transfected cells.

compared with those of corresponding empty vector transfected cells and parental cells ($P < 0.01$). There was no significant difference in the cell migration rate between empty vector transfected cells and parental cells ($P > 0.05$). These results suggest that MAGE-D1 can inhibit the migration of breast cancer cells.

Effect of MAGE-D1 plasmid transfection on invasion of breast cancer cells. To investigate the role of MAGE-D1 gene in invasion of breast cancer cells, we performed *in vitro* invasion assays in MDA-MB-231 cells, a highly metastatic breast cancer cell line (Fig. 5). The number of invading cells of MAGE-D1/MDA-MB-231 was 36.1% less than that of vector/MDA-MB-231 cells. There was no significant difference in the cell invasion between empty vector transfected cells and parental cells ($P > 0.05$). These results suggest that MAGE-D1 can inhibit the invasion of breast cancer cells.

Effect of MAGE-D1 plasmid transfection on the expression of proliferation- and invasion-related molecules. In an attempt to elucidate the mechanism of MAGE-D1 in suppressing cellular growth and invasion, the protein expressions of p53, p21, E-cadherin, β -catenin, MMP-2 and MMP-9 were assessed in MCF-7 and MDA-MB-231 cells. Western blotting showed that protein expression of E-cadherin was up-regulated, and β -catenin was down-regulated in MAGE-D1/MCF-7 cells when compared with vector/MCF-7 cells (Fig. 6A, C), but not altered in MAGE-D1/MDA-MB-231 cells (Fig. 6B, D). Protein expressions of p53 and p21 were significantly increased in MAGE-D1/MDA-MB-231 cells, relative to the vector/MDA-MB-231 cells (Fig. 6B, D), but not changed in MAGE-D1/MCF-7 cells (Fig. 6A, C). The expressions of MMP-2 and MMP-9 were undetectable in MCF-7 cells, and remained almost unchanged in MAGE-D1/MDA-MB-231 cells when compared with vector/MDA-MB-231 cells (Fig. 6B, D). This result indicated that the inhibition effect of MAGE-D1 on cellular proliferation was associated with p53 pathway, while its effect on cellular invasion was related to E-cadherin and β -catenin.

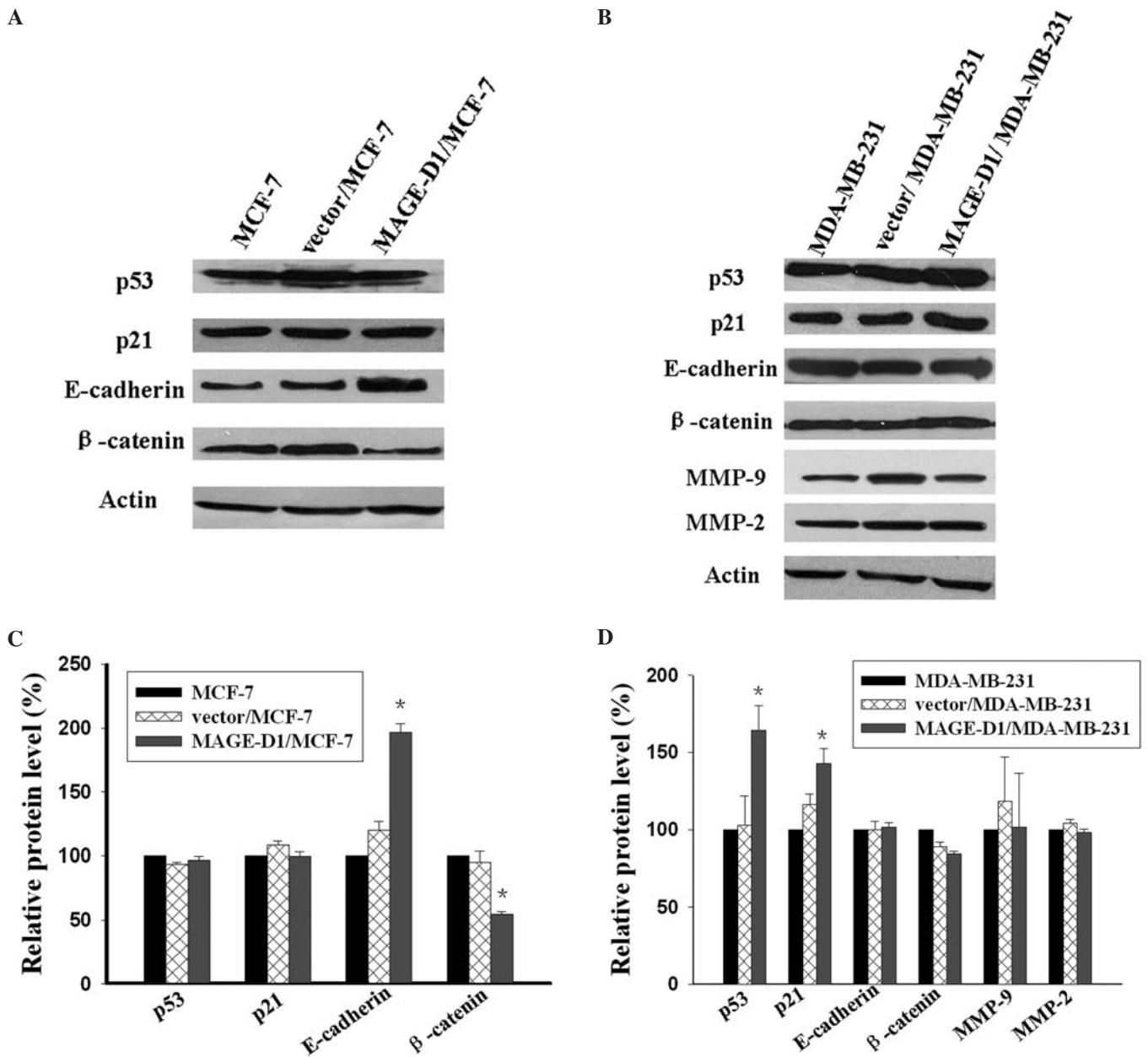


Figure 6. Effect of MAGE-D1 plasmid transfection on the expression of proliferation- and invasion-related molecules. (A and B) Western blot analysis using antibodies against p53, p21, E-cadherin, β-catenin, MMP-9 and MMP-2. The actin serves as a loading control. Similar experiments were repeated at least twice. (C and D) Relative expression levels of these proteins. For quantification, the intensity of each band was evaluated by densitometry using the quantity-one image analysis software (Bio-Rad). The relative protein value is presented as a ratio of the tested protein's signal divided by its corresponding actin. *P<0.01 vs. vector transfected cells.

Discussion

In this study, for the first time we report that MAGE-D1 transfection blocks proliferation, migration and invasion of breast cancer cells. In recent years, there is increasing evidence to suggest that MAGE-D1 has anti-proliferative activity. MAGE-D1, as a BRCA2 binding protein, acts synergistically with BRCA2 in suppressing the proliferation of mammary epithelial cells (4). *In vitro*, MAGE-D1 showed significant anti-proliferative effects in the kidney epithelial cells HEK293, human hepatocellular carcinoma cells HepG2 and human osteosarcoma cells U2OS (25). MAGE-D1 also interacted with its structural homologous, necdin, which is a potent growth suppressor and expressed predominantly in

postmitotic cells such as neurons and skeletal muscle cells, and induced growth arrest (14). In this study, MTT assay and cell counting consistently showed that MAGE-D1 transfection could effectively inhibit the proliferation of MDA-MB-231 and MCF-7 breast cancer cells. To further investigate the probable mechanism of anti-proliferative efficacy of MAGE-D1, we examined the effect of MAGE-D1 transfection on cell cycle distribution, apoptosis and p53 pathway. Our results showed that MAGE-D1 up-regulated p53 and p21 expression in MDA-MB-231 cells, but did not affect cell cycle distribution and apoptosis in MDA-MB-231 and MCF-7 cells. Wen *et al* found that MAGE-D1 suppressed cell growth in HEK293, U2OS and HepG2 cells through p53-dependent pathway. The cell cycle arrest occurred

at the G2/M and G1 stage (25). Salehi *et al* demonstrated that MAGE-D1 mediated G1 arrest in sympathetic precursor cells through p75NTR (15). MAGE-D1 is also involved in apoptosis by interacting with p75 neurotrophin receptor, axon guidance receptor UNC5H, Ror receptor kinases, inhibitors of apoptosis proteins (IAP), ubiquitin ligase praja1, and anti-apoptotic factor Che-1 (16-20). Under BMPR stimulation, MAGE-D1 facilitated the formation of a TAK1-TAB1-XIAP-MAGE-D1 complex that activated p38, driving cellular apoptosis in neural progenitor cells (26). Salehi *et al* reported that MAGE-D1 could induce caspase activation and cell death through the c-Jun N-terminal kinase (JNK)/c-Jun pathway (27). However, in agreement with our findings, MAGE-D1 did not induce apoptosis in the kidney epithelial cells HEK293, human hepatocellular carcinoma cells HepG2 and human osteosarcoma cells U2 OS (25). One possible explanation is that MAGE-D1 has distinct functions in different cells. Alternatively, MAGE-D1 may not be involved in cell cycle regulation and apoptosis of MCF-7 and MDA-MB-231 breast cancer cells.

Tumor cell migration and invasion are critical factors for malignant tumor metastasis, which is a multiple process and is regulated by many genes. E-cadherin acts as intercellular glue and mediates homophilic, calcium-dependent cell-cell adhesion, and its cytoplasmic portion binds to β -catenin, which connect the adhesion complex to the actin cytoskeleton (28). The E-cadherin/catenin-mediated cell adhesion system is known to act as an 'invasion suppressor system'. In this study, wound healing assay demonstrated that MAGE-D1 inhibited cellular migration of breast carcinoma cells. Boyden chamber assay showed that MAGE-D1 effectively blocked the cell invasion of the highly metastatic cell line MDA-MB-231. Western blotting revealed that MAGE-D1 up-regulated E-cadherin expression and down-regulated β -catenin expression in breast carcinoma cells. Similarly, Xue *et al* showed that human MAGE-D1 can inhibit U2 OS cell-to-cell adhesion by disrupting the E-cadherin/ β -catenin complex (11). Shen *et al* demonstrated the suppression of MAGE-D1 on cell migration, invasion, adhesion and *in vitro* tube formation in either normoxic or hypoxic conditions (12). MAGE-D1 has also been reported to suppress metastasis of melanoma and pancreatic cancer *in vitro* and *in vivo*, which indicates that human MAGE-D1 may be involved in cell adhesion and invasion (21). The metastatic process requires the degradation of the extracellular matrix both at the primary tumor site and at the secondary colonization site. This degradation process is dependent on the activity of specific endopeptidases, the matrix metalloproteinases (MMPs) (29). MMP2 and MMP-9 expression has been related to the invasive property of a variety of cancers including breast carcinoma (30,31). Our data showed that MDA-MB-231, a highly metastatic breast cancer cell line, expresses higher level of MMP9 than MCF-7, a relatively low metastatic breast cancer cell line. While in this study, MAGE-D1 did not alter the MMP-2 and MMP-9 expression, suggesting these molecules may not be involved in MAGE-D1 mediated suppression of invasion.

In summary, this study suggests that MAGE-D1 play important roles in the regulation of cell proliferation, migration and invasion of breast cancer cells. Enhanced expression of MAGE-D1 by gene transfer could reverse the malignant

phenotypes of breast cancer cells. MAGE-D1 may be a potential therapeutic target for breast cancer.

Acknowledgements

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