PTEN-mRNA engineered mesenchymal stem cell-mediated cytotoxic effects on U251 glioma cells

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Abstract. Mesenchymal stem cells (MSCs) have been considered to have potential as ideal carriers for the delivery of anticancer agents since the capacity for tumor-oriented migration and integration was identified. In contrast to DNA-based vectors, mRNA synthesized *in vitro* may be readily transfected and is mutagenesis-free. The present study was performed in order to investigate the effects of phosphatase and tensin homolog (PTEN) mRNA-engineered MSCs on human glioma U251 cells under indirect co-culture conditions. PTEN-bearing mRNA was generated by *in vitro* transcription and was transfected into MSCs. The expression of PTEN in transfected MSCs was detected by immunoblotting, and the migration ability of MSCs following PTEN-bearing mRNA transfection was verified using Transwell co-cultures. The indirect co-culture was used to determine the effects of PTEN-engineered MSCs on the viability of U251 glioma cells by luminescence and fluorescence microscopy. The synthesized PTEN mRNA was expressed in MSCs, and the expression was highest at 24 h subsequent to transfection. An enhanced migration rate was observed in MSCs transfected with PTEN mRNA compared with non-transfected MSCs (P<0.05). A significant inhibition of U251 cells was observed when the cells were cultured with conditioned medium from PTEN mRNA-engineered MSCs (P<0.05). The results suggested that anticancer gene-bearing mRNA synthesized *in vitro* is capable of being applied to a MSC-mediated anticancer strategy for the treatment of glioblastoma patients.

Introduction

Gliomas are recognized as highly malignant and intractable cancers and are the most common type of primary brain tumors throughout the world (1). The complex cellular composition, diffuse invasiveness and capacity to escape conventional therapies have been challenging and have hampered progress towards an effective treatment (2,3). A major obstacle limiting the effectiveness of conventional therapies for gliomas and other types of cancer, is the lack of tumor specificity (4,5). Therefore, exploring the therapeutic strategies that specifically target tumor tissues is critical.

The mesenchymal stem cell (MSC)-mediated strategy has demonstrated considerable potential for the development of clinically meaningful patient-tailored anticancer therapies (6-9). The tumor-directed migration and incorporation of MSCs have been demonstrated in a number of preclinical studies *in vitro,* using Transwell migration assays, and *in vivo,* using animal tumor models (10-12). Directed at the tumor microenvironment, engineered MSCs are able to express or release anticancer substances that may constantly act on the adjacent tumor cells (13). As a direct attacker, an anticancer gene that was pre-engineered on MSCs plays a critical role for the action in this system (14). Plasmid DNA transfection and viral vector transduction are widely used in gene therapy-associated studies (15-19). These methodologies have the potential hazards of genomic recombination or insertional mutagenesis, which is a significant problem when this technology is applied to gene therapy (20,21). Therefore, an approach that carries anticancer genes using MSCs, without genetic modification and without risks for clinical applications, may be desired for anticancer medicine. Recently, a novel stabilized mRNA construct has become a more attractive alternative to the most commonly used DNA-based plasmid (pDNA) (22). Synthesized mRNA *in vitro* has been

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Table I. Primers for *in vitro* synthesized PTEN mRNA.

PTEN mRNA	Forward primer	Reverse primer
PTEN ORF	F1: ATGAAGTTTCCTTCTCAACTTC	R1: TCAGACTTTTGTAATTTGTGTATG
	F2: CGCGGATCCGCCACCATGAAGTTTCC	R2:CCGCTCGAGTCAGACTTTTGTAATTT
5' Splint	GTACTGCTCCTCGCCGTTCATGGTGGC TCTTATATTTCTTCTTACTC	
3' Splint	CCCGCAGAAGGCAGCTTACTCATCGTG GTTCCTGCGGCC	
5' UTR	TTGGACCCTCGTACAGAAGCTAATACG ACTCACTATAGGGAAATAAGAGAGAAA AGAAGAGTAAGAAGAAATATAAGAGCC ACC	
3' UTR	GCTGCCTTCTGCGGGGCTTGCCTTCTG GCCATGCCCTTCTTCTCTCCCTTGCAC CTGTACCTCTTGGTCTTTGAATAAAGC CTGAGTAGGAAGTGAGGGTCTAGAACT AGTGTCGACGC	
Ligation product PCR	TTGGACCCTCGTACAGAAGCTAATACG	GCGTCGACACTAGTTCTAGACCCTCA
Tail PCR	TTGGACCCTCGTACAGAAGCTAATACG	T_{120}CTTCCTACTCAGGCTTTATTCAAA

PTEN, phosphatase and tensin homolog; mRNA, messenger RNA; ORF, open reading frame; UTR, untranslated region; PCR, polymerase chain reaction.

widely used to generate induced pluripotent stem cells or induce cell reprogramming (23-26).

Phosphatase and tensin homolog (PTEN) functions as the important negative regulator of PI3K-AKT-mTOR pathway in controlling cells apoptosis (27-29). The activity of PTEN is lost by deletions, mutations or promoter methylation at a high frequency in numerous human cancers (30). Therefore, restoring PTEN function in cancer cells may break down the PTEN mutation-dependent cancer cell growth, termed onco-gene addiction, and demonstrates considerable promise for cancer therapy. The present study describes an experimental strategy that inhibits malignant glioma U251 cells using a conditioned medium (CM) from PTEN-engineered MSCs through the use of *in vitro* synthesized PTEN mRNA. The strategy has initiated the application of advantageous PTEN mRNA in the field of cancer research. The novel synthetic modified PTEN mRNA possesses great potential for use as an effective therapeutic candidate for glioblastoma patients.

Materials and methods

Cells and culture conditions. MSCs were isolated from healthy human pancreatic ductal tissue and expanded *ex vivo* as previously described (31). Briefly, human pancreases were obtained with full informed consent from adult heart-beating cadaver organ donors through the organ procurement program at the British Columbia Transplant Society (Vancouver, Canada). A total of 5 pancreatic ductal tissue samples were collected between July 2011 and November 2011 at Vancouver General Hospital (Vancouver, Canada). The pancreatic ductal tissue taken from the Ricordi® Chamber (Biorep Technologies, Inc., Miami, FL, USA), which was used for islet isolation, was utilized as the starting material for MSC isolation. The

human glioma U251 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) to be used as target cells in the present study. U251 cells were maintained as suggested by ATCC, and the culture conditions were kept consistent with those of the MSCs. In order to track the viability in a timely manner, the U251 cells were pre-labeled with luciferase using the pGL4.51[luc2/CMV/Neo] vector (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

In vitro synthesis of PTEN mRNA and MSC transfection. The *in vitro* transcription template construction and RNA synthesis is schematized in Fig. 1. The oligonucleotide sequences used in the construction of *in vitro* transcription templates are shown in Table I. The human 5'-UTR with Kozak and 3'-UTR sequences were synthesized commercially by Integrated DNA Technologies (Coralville, IA, USA), sub-cloned into a pcDNA3.3 plasmid and termed the pcDNA 3.3-TOPO TA vector. As previously described (23), plasmid inserts were excised using restriction enzyme digestion and used to template tail polymerase chain reaction (PCR). RNA was synthesized using the Ambion MEGAscript T7 kit (Thermo Fisher Scientific, Waltham, MA, USA). The ribonucleoside blend was composed of anti-reverse cap analogs (New England Biolabs, Inc., Ipswich, MA, USA) and adenosine triphosphate, guanosine triphosphate, 5-methylcytidine triphosphate and pseudouridine triphosphate (TriLink Biotechnologies, San Diego, CA, USA). Reactions were incubated for 5 h at 37˚C and followed by DNase treatment, according to the manufacturer's protocol. Then, the reactions were treated with Antarctic Phosphatase (New England Biolabs, Inc.) for 2 h at 37˚C in order to remove residual 5'-triphosphates. The synthesized RNA was purified with Ambion MEGAclear
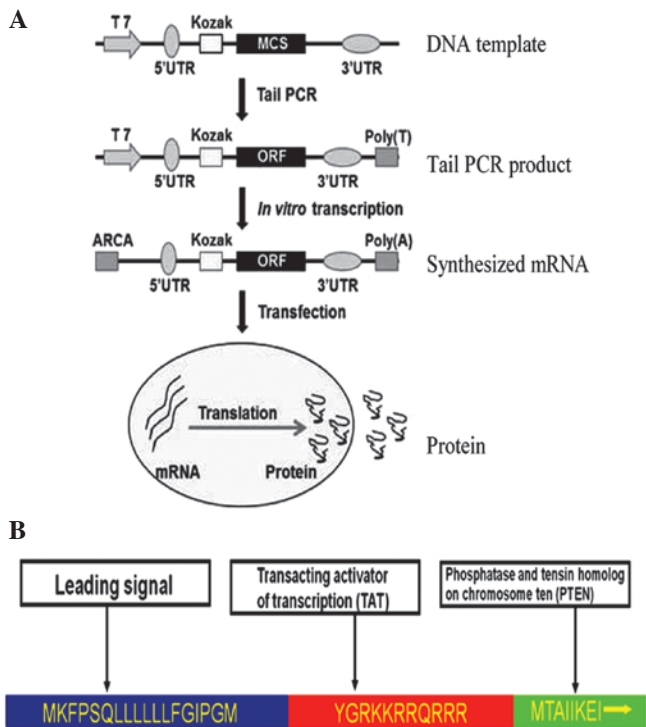
Figure 1. Flow charts of PTEN mRNA synthesis *in vitro* and the design of the ₛₜPTEN fusion protein. (A) Schematic diagram of RNA synthesis *in vitro*. (B) Design of the ₛₜPTEN fusion protein. PTEN, phosphatase and tensin homolog; ₛₜPTEN, secretory phosphatase and tensin homolog.

spin columns (Thermo Fisher Scientific) and quantified using NanoDrop (Thermo Fisher Scientific). RNA transfection was performed using TransIT-mRNA (Mirus Bio, Madison, WI, USA). RNA was diluted in Invitrogen Opti-MEM basal media (Thermo Fisher Scientific), and the Boost reagent (Mirus Bio LLC, Madison, WI, USA) and TransIT-mRNA were added sequentially according to the manufacturer's protocol. Subsequent to 2 min incubation at room temperature (RT), the RNA-lipid complexes were delivered to culture media in the culture plates. The plates were then returned to the incubator and PTEN expression was analyzed 12, 24 and 36 h later using western blot analysis.

Viability assay of tumor cells in indirect co-culture. The cytotoxic effects of PTEN-engineered MSCs (MSC^PTEN) on the proliferation of malignant glioma cells were assessed under indirect co-culture conditions. The luciferase gene transfected U251 cells were plated at a density of 5,000 cells/well in a Falcon 96-well plate (BD Biosciences, Franklin Lakes, NJ, USA) in 100 μl of minimal essential medium (MEM) on day 0. The cells were cultured using CM that was collected from the cultures of native MSCs (CM_control) and MSC^PTEN (CM_PTEN) on day 1. The culture volume was 100 μl with five different ratios of MSC^PTEN to CM from MSC_control, as follows: 0, 25, 50, 75 and 100%. On days 0, 3 and 6, 1 μl (0.15 mg/ml) D-luciferin (Caliper Life Sciences, Waltham, MA, USA) was added into each well and the cells were incubated in the same culture conditions for an additional 10 min. Luminescence was measured using the IVIS Spectrum System (Caliper Life Sciences). The media were replaced every 36 h and the measurements of every group were repeated three times.

Migration assay of MSCs with Transwell co-culture system. A cell migration assay was performed, as previously described (32). U251 cells were placed on the plastic surface of 24-well plates (1x10^5 cells/well). On the following day, the wild-type MSCs and MSC^PTEN in serum-free media were seeded onto the 8 μm microporous membranes of the Transwell insert (Corning Incorporated, Corning, NY, USA). MSCs were incubated for 48 h at 37°C and in a 5% CO_2 atmosphere. The inserts were washed with phosphate buffered saline (PBS) and the upper surface of the membrane was scraped gently using a cotton swab to remove non-migrated cells. Then, the membranes were fixed with 95% ethanol (Sangon Biotech Co., Ltd., Shanghai, China) and stained using 0.1% crystal violet (Sangon Biotech Co., Ltd.). The average number of migrated cells was assessed by counting five randomly selected microscopic fields at x100 magnification. All experiments were repeated at least three times. To detect the migration status of the MSCs with normal cells, U251 glioma cells were replaced with native MSCs on the plastic surface of the 24-well plates.

Immunoblotting. Immunoblotting was used to detect the cellular expression of MSCs. Briefly, the MSCs were washed with PBS three times and collected using cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Cell lysates were incubated on ice for 30 min. The protein concentration was determined using bicinchoninic acid protein assay reagents (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Equal amounts of protein (50 μg/sample) were loaded on each lane and separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to nitrocellulose membranes (Beyotime Institute of Biotechnology). The membrane was incubated in blocking buffer for 1 h at RT and then incubated overnight at 4°C with monoclonal rabbit anti-mouse PTEN primary antibodies (cat. no. 217702; dilution, 1:1,000; R&D Systems, Inc., Minneapolis, MN, USA). The blots were rinsed with Tris-buffered saline and Tween-20 (EMD Millipore, Billerica, MA, USA) three times, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat. no. A0216; dilution, 1:1,000; Beyotime Institute of Biotechnology) for 60 min and detected by chemiluminescence using ECL Hyperfilm (EMD Millipore). The CM samples were filtered through a 0.22 μm membrane (EMD Millipore) and equally concentrated using a 10,000 molecular weight cut-off (catalog no., 42406; EMD Millipore). Then, PTEN was detected in the CM.

Fluorescence microscopy. The cell viability was also detected using an Invitrogen LIVE/DEAD Viability/Cytotoxicity assay kit (Thermo Fisher Scientific), according to the manufacturer's protocol, but with a slight modification (10). Briefly, a total of 1x10^5 U251 cells were plated onto 24-well plates in 500 μl of MEM on day 0. The media were replaced with 50 or 100% CM on day 1. On day 4, the cultures were washed twice with PBS. Freshly prepared working solution containing 1μM calcein acetoxymethyl and 2 μM ethidium homodimer-1 (Thermo Fisher Scientific) was then added directly to the cultures, with 250 μl solution added per well on 24-well plates, and the wells were incubated at RT for 10 min in the dark. The images were captured using a fluorescence microscope (IX71; Olympus
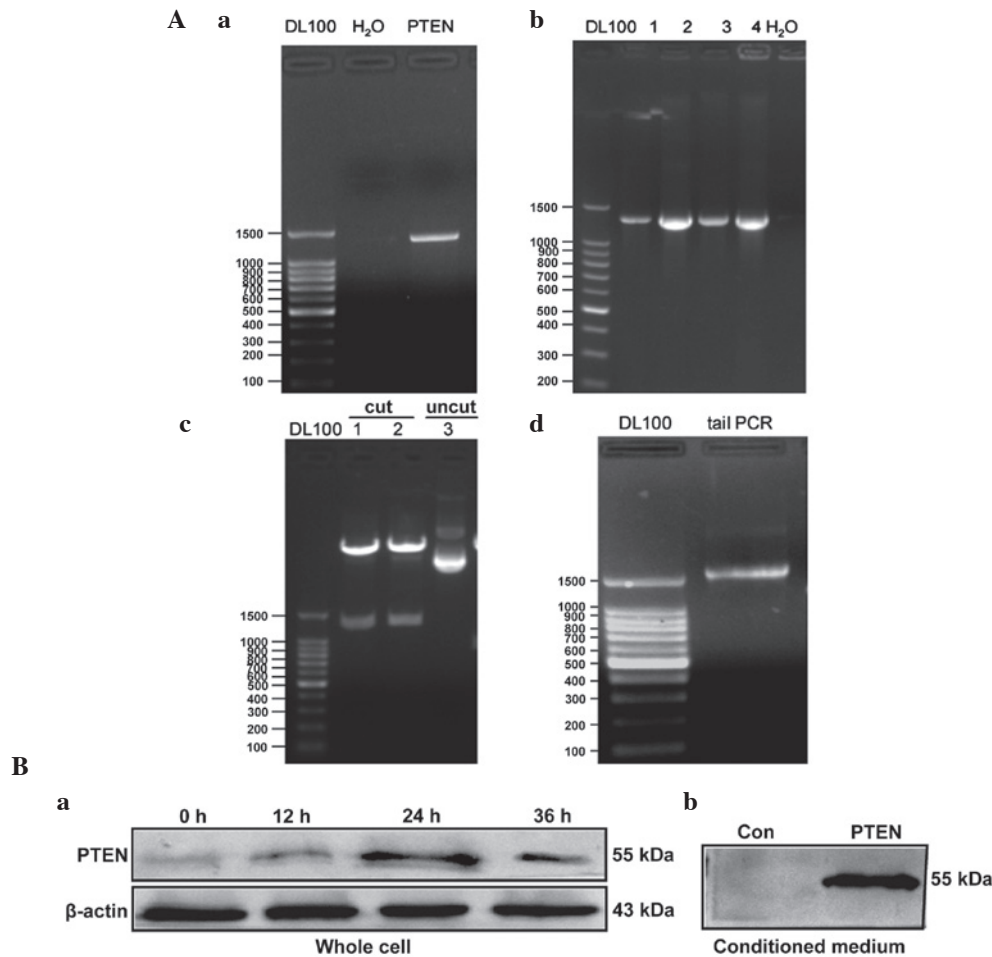
Figure 2. *In vitro* synthesis of PTEN mRNA and transfection into MSCs. (A) Analysis of synthesized PTEN mRNA by agarose gel electrophorsis. (a) Analysis of the PTEN sequence PCR product. The PTEN sequence was inserted into the pcDNA 3.3-TOPO TA vector by (b) single colony PCR and (c) restriction enzyme digestion. (d) PTEN tail PCR product. Lanes 1-4 contain different clones. (B) Immunoblotting analysis of PTEN expression in (a) cell lysates and (b) conditioned media. H_2O acted as the PCR template. DL100, 100-bp DNA ladder maker; PTEN, phosphatase and tensin homolog; mRNA, messenger RNA; MSCs, mesenchymal stem cells; PCR, polymerase chain reaction; Con, control.

Corporation, Tokyo, Japan) and the associated analysis was performed using ImageJ 1.45 k software (National Institute of Health of USA, Bethesda, MD, USA).

Statistical analysis. Numerical data were expressed as the mean ± standard error. Statistical differences between the means for the various groups were evaluated using Prism 4.0 software (GraphPad software, San Diego, CA, USA) using the Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PTEN-bearing mRNA synthesis and expression in MSCs. Anticancer gene-bearing mRNA was constructed as illustrated in Fig. 1A. In order to make the PTEN product secretory (stPTEN), a leading sequence that corresponded to an 18 amino acid segment (sequence, MKFPSQLLLLLLF-GIPGM) was introduced into the DNA template. A transacting activator of transcription (TAT; sequence, YGRK-KRRQRRR) was inserted into the PTEN template, to act at the transmembrane (Fig. 1B). The stPTEN DNA template was cloned from previously constructed pDsRed1-TAT-PTEN

vectors (Fig. 2Aa) (33) and the PCR product of the stPTEN sequence was then inserted into the pcDNA 3.3-TOPO TA vector. The inserted DNA sequence was confirmed by an analysis of the colony using PCR (Fig. 2Ab), restriction enzyme digestion (Fig. 2Ac) and sequencing (data not shown). The correct plasmid insert was excised by restriction enzyme digestion (using *BAm*HI and *Xho*I; Takara Biotechnology Co., Ltd., Dalian, China) and used to template tail PCR (Fig. 2Ad). As shown in Fig. 2B, the expression of PTEN was clearly enhanced in transfected MSCs. The endogenous PTEN was observed in control MSCs with a slightly varied size, the maximum expression of transfected PTEN mRNA was observed at 24 h post transfection, and subsequently decreased at 36 h. The stPTEN was also detectable in the corresponding CMs.

Effect of mRNA transfection on MSC migration. MSC migration was analyzed using the Transwell system. Subsequent to 48 h of co-culture, a considerable number of cells migrated across the microporous membrane towards the U251 cells (Fig. 3A). However, few cells migrated towards the normal MSC control cells (Fig. 3B). Notably, the transfection of PTEN-mRNAs significantly enhanced the migration of
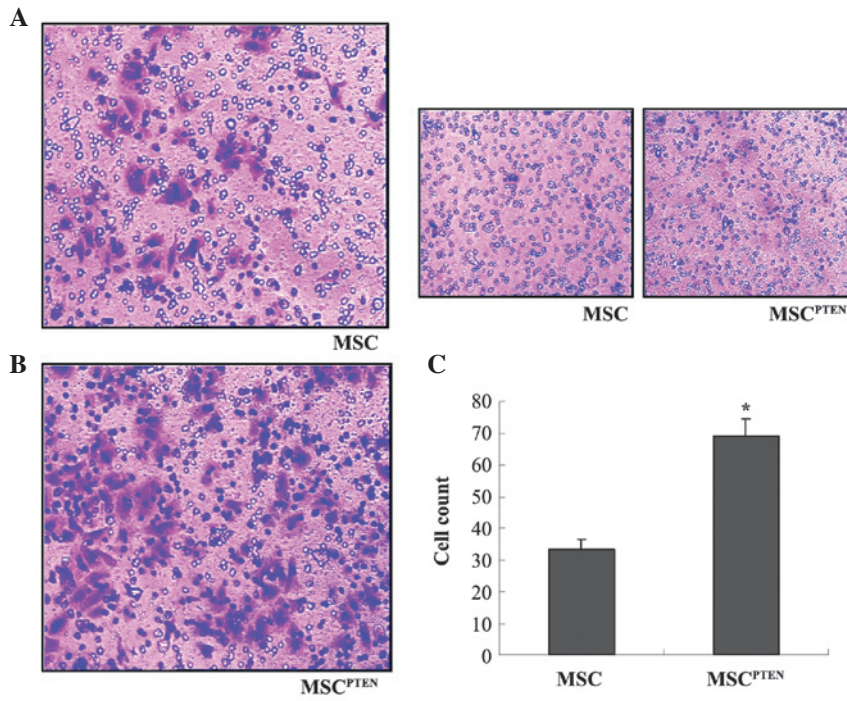
Figure 3. *In vitro* migratory ability of MSCs. (A) The migratory ability of wild-type MSCs and MSC[PTEN] toward U251 glioma cells using a Transwell co-culture system. Representative photomicrographs of the stained membrane indicates migrated cells (magnification, x100). (B) The migration of MSCs was tested using wild-type MSCs as control cells (magnification, x100). (C) Summary of three independent experiments was presented as mean ± standard error of the mean. The cell number of each filter membrane was obtained from the average of five randomly selected microscopic fields. *P<0.05 vs. wild-type MSCs. MSCs, mesenchymal stem cells; PTEN, phosphatase and tensin homolog; MSC[PTEN], PTEN-engineered MSCs.
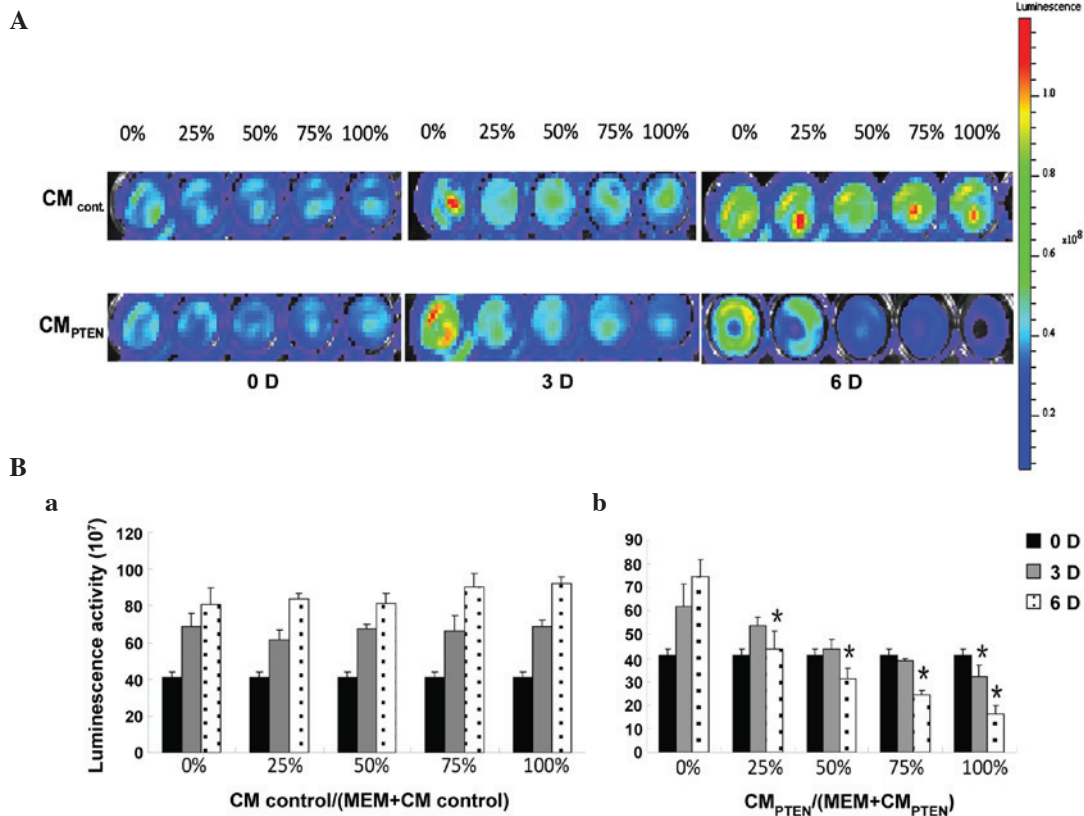

Figure 4. Assessment of U251 cell viability using bioluminescence determination. (A) Representative measurement of luminescence intensity. Cell culture medium is indicated on the left side of the graph. CM ratios and time points are labeled at the top and bottom of the images respectively. The luminescence intensity of each well was determined by IVIS Spectrum System, 10 min subsequent to adding D-luciferin. Luminescence scale: Color scale: Min = 6.31x10^6; Max = 1.19x10^8. (B) Summary of U251 cell viability. U251 cells were co-cultured with (a) CM_control and (b) CM_PTEN. The relative cell viability is represented as luciferase activity. Data are presented as mean ± standard error of the mean. *P<0.05 vs. control (0%) at the same time point. CM, conditioned media; PTEN, phosphatase and tensin homolog; CM_CONTROL, CM collected from the cultures of native MSCs; CM_PTEN, CM collected fom the cultures of PTEN-engineered MSCs.
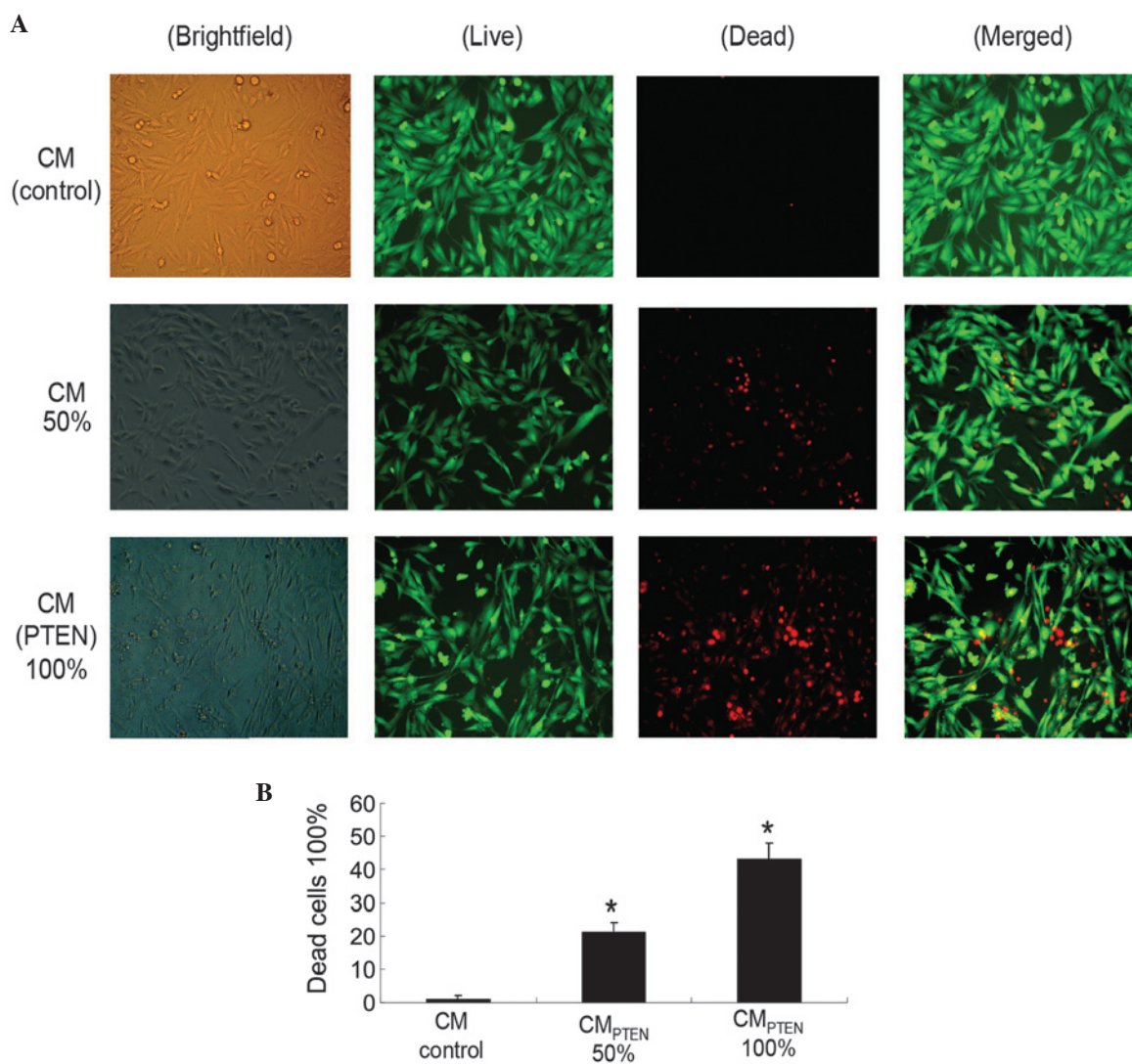
Figure 5. U251 cell viability in indirect co-cultures. (A) Cells were incubated in various CMs (indicated on the left side of the graph) at various ratios (indicated at the top). LIVE/DEAD staining was performed on day 4 following the initiation of the indirect co-culture (orginal magnification, x400). (B) Summary of cell viability of indirect co-cultures. Data are expressed as the mean ± standard error of the mean for three independent experiments. *P<0.05 vs. control. Brightfield, whole population of cells attached to the culture surface; Live, live cells stained with calcein in green; Dead, dead cells stained with ethdium homodimer-1 in red; Merged, merged images; CM, conditioned media, PTEN, phophatase and tensin homolog; CM$_{CONTROL}$, CM collected from the cultures of native MSCs; CM$_{PTEN}$, CM collected fom the cultures of PTEN-engineered MSCs.

MSCs towards the U251 glioma cells compared with control MSCs (Fig. 3C).

Effect of PTEN-engineered MSCs on the viability of U251 glioma cells. The indirect co-culture was used to determine the effects of PTEN-engineered MSCs on the viability of U251 glioma cells by luminescence. As shown in Fig. 4A, the intensity of luminescence in U251 cells decreased with the increased incubation time and CM ratio. All test results are summarized in Fig. 4B. Starting at a low CM ratio of 25%, the cells incubated with CM$_{PTEN}$ revealed significant cell death at day 6 (47.7%; P<0.05). However, significant cell death (P<0.05) started to appear at day 3, at a CM ratio of 100%. The ratio of CM$_{PTEN}$-induced cell death at day 3 was not statistically different from day 0.

MSC^{PTEN}-mediated U251 cell death in indirect co-cultures. CM-induced U251 cell death was also examined on day 4 by fluorescence microscopy (IX71; Olympus Corporation) subsequent to LIVE/DEAD staining. Two CM ratios, 50 and 100%, were used in this component of the study. As shown in Fig. 5, the cell death was proportionally associated with CM$_{PTEN}$. The dose-dependent cell death indicates that MSC^{PTEN}-derived PTEN is an important mediator responsible for U251 cell death during indirect co-culture. Marked cell death was not detected in the CM obtained from the native MSCs in the current experimental conditions.

Discussion

Malignant glioma is one of the most challenging cancers to treat successfully, mainly due to the particularity of its location and biological characteristics, including the infiltrative nature, resistance to apoptosis, propensity for recurrence and resistance to conventional therapies (3). Since the identification of the tumor-oriented migration capacity of MSCs, the application of

specific anticancer gene-engineered MSCs has demonstrated potential for the development of targeted therapy of malignant gliomas (34-36). While MSCs may carry anticancer genes with various viral or plasmid vectors, these methods may not be used in clinical applications (37).

Synthetic mRNA *in vitro* is a novel technique that presents a safe, efficient strategy for gene therapy (25). The use of synthetic mRNA completely removes the risk of modifying the host genome (38-39), and the safety of synthetic mRNA has been demonstrated in clinical studies (40). Therefore, the synthetic mRNA-based strategy is a simple and non-integrating approach for the application of specific anticancer gene-engineered MSCs.

The loss of PTEN expression in a wide range of cancer cells, including gliomas, reflects the importance of PTEN in the maintenance of cancer cell survival, with >50% of human glioblastoma cases exhibiting loss of expression (41). PTEN function restoration may inhibit cancer cell growth and may induce cell death under certain circumstances, such as loss of PTEN expression (28-30). In consideration of the potential clinical applications, $_{st}$PTEN mRNA was synthesized *in vitro* and used in the present MSC-mediated anticancer study. As demonstrated in Fig. 2B, the synthesized PTEN mRNA was expressed in MSCs with the peak activity at 24 h following transfection. The expression is consistent with a previous study, which used a DNA-based vector in the same cell model (33).

In the present study, the tumor cell-oriented migration of MSCs, which were engineered with DNA-based anticancer gene expression vectors, was investigated using the xCELLigence system in pancreatic cancer cells and glioma DBTRG cells (33,42). In order to make the strategy clinically practical, the effects of synthesized mRNA transfection on the migration ability of MSCs was detected. As shown in Fig. 3, the migration of MSCs was not obstructed by PTEN mRNA transfection. Notably, the migration of MSCs towards the U251 cells was significantly increased by transfected PTEN mRNA. The underlying mechanisms require additional studies in the future. The results of the present study suggest that synthesized mRNA may be applied to the MSC-mediated anticancer strategy.

To the best of our knowledge, the present study is the first demonstration that synthetic modified PTEN mRNA engineered MSC-mediated cytotoxic effects on glioma cells. In the present study, the cytotoxic effects of CM from MSC^{PTEN} on U251 cells were examined using a luminescence technique and fluorescence microscopy. Under the current indirect co-culture conditions, U251 cells were sensitive to CM$_{PTEN}$ (Fig. 4). The significant cytotoxicity was observed on day 6 and at a CM ratio of 50%. The LIVE/DEAD assay possessed the advantage of being straightforward, and reflected the intact status of the detected cells at any time point. As shown in Fig. 5, dose-responsible death of U251 glioma cells revealed the anticancer effect of MSCs that were transfected with PTEN-mRNAs. However, the drawback of the LIVE/DEAD assay is that it only applies to the cells that remain on the culture surface during staining. The detached cells, the majority of which are dead cells, are not included in the assessment. Multiple assessments and *in vivo* experiments are required in additional studies.

In the present study, a safe and efficient strategy of mRNA-based gene transfer was successfully applied in the MSCs-mediated cytotoxicity of tumor cells. The approach of MSC-mediated gene therapy caused a dual-targeted killing effect to the tumor, mainly due to the tropism characteristics of MCSs and engineered anticancer agents. mRNA-based gene transfer may also kill tumor cells locally and consistently. The methodology used in the present study also holds the potential to use the MSCs of the patients and to switch the tumor attackers corresponding to the individual condition. In conclusion, the present study provides an essential foundation for additional *in vivo* and potentially clinical anticancer studies using synthesized mRNAs.

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