Optimization and establishment of RNA interference-mediated knockdown of the progestagen-associated endometrial protein gene in human metastatic melanoma cell lines

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Abstract. Progestagen-associated endometrial protein (PAEP), also termed glycodelin, is a 28-kDa glycoprotein of the lipocalin superfamily that is expressed in a variety of tumors, including gynecological tumors, lung cancer and melanoma. To determine the biological functions of the PAEP gene in tumor development and progression, the production of transient and stable PAEP knockdown cell models is required. In the present study, RNA interference technology was used to silence PAEP gene expression in melanoma and transfection was screened for and the conditions were optimized using fluorescence microscopy, flow cytometry, qPCR and western blot analysis. The results of the present study showed that the transient transfection of melanoma cells with 100 nmol/l PAEP siRNA or lentiviral PAEP small hairpin RNA (shRNA) [multiplicity of infection (MOI), 100 pfu/cell] efficiently knocked down target gene expression. To establish stable PAEP knockdown cell lines, melanoma cells were infected with a low MOI (10 pfu/cell) of lentiviral particles expressing PAEP shRNA. Following puromycin screening, the PAEP gene knockdown efficiency was demonstrated to be >80% in 624-Mel and 624.38-Mel cell lines, which was validated by western blot analysis. Therefore, melanoma cell lines with stable knockdown of PAEP were successfully established and may be used as effective cell models to study the biological functions of the PAEP gene in melanoma.

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Introduction

Progestagen-associated endometrial protein (PAEP) is a secreted glycoprotein, which was first isolated from human placenta, amniotic fluid, decidua of pregnancies and seminal plasma (1). It has been demonstrated to be important in a number of physiological processes, such as embryonic implantation, immunotolerance, contraception and gland differentiation. In recent years, several studies have demonstrated that PAEP is abnormally expressed in various types of tumors, such as endometrial carcinoma, ovarian cancer, breast cancer (2-5), lung cancer (6) and melanoma (7). Transfection of PAEP cDNA into the MCF-7 breast cancer cell line results in marked changes in cell growth behavior, with the suppression of proliferation and formation of acinar structures (8), suggesting that PAEP inhibits tumor cell growth and promotes cell differentiation as a tumor suppressive factor. Song *et al* (9) demonstrated that PAEP is also involved in neovascularization during tumor growth (9). However, our previous study presumed that PAEP is a tumor promoter in melanoma (10), suggesting contradictory results as compared to other studies.

Melanogenesis is a result of the malignant transformation of neural crest-derived melanocytes (11), and melanoma is one of the most aggressive forms of human cancer. Once metastasized, it is difficult to treat and is associated with high mortality rates. PAEP has been shown to be highly expressed in melanoma tissues and the majority of the melanoma cell lines tested in the literature thus far. Therefore, the present study aimed to establish melanoma cell lines with low PAEP gene expression to investigate their cytological and genetic functions in tumorigenesis and tumor development.

In the present study, RNA interference (RNAi) technology was utilized to silence PAEP gene expression, using transfection with PAEP-specific small interfering RNA (siRNA) or infection with lentiviral vector-mediated small hairpin RNA (shRNA) to lead to the transient or stable knockdown of PAEP gene expression in melanoma cells. The cell lines obtained from this study may be utilized to clarify the function of the PAEP gene.

Materials and methods

Cell lines and culture. Four cell lines (MCC69B, 624-MEL, 624.38-Mel and FEMX-I lines) originally procured from melanoma patients were used. MCC69B cells were isolated from a distant metastasis, the 624-Mel and 624.38-Mel metastatic melanoma cell lines were obtained from the National Cancer Institute (NIH). FEMX-I, provided by Oystein Fodstand from Norwegian Radium Hospital (Oslo, Norway), originated from a lymph node metastasis in a patient, uniquely and selectively produced extrapulmonary metastases following intravenous (i.v.) injection of cells prepared from xenografts into adult, nude mice (12). All the melanoma cells were maintained in RPMI-1640 culture media (Hyclone, Thermo Scientific, Beijing, China) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific).

Design of siRNA. SiGLO green is a fluorescent marker that is used to monitor infection efficiency. In the present study, four duplex siRNAs targeting PAEP mRNA were designed. The target sequences were as follows: siPAEP9, TCA ACT ATA CGG TGG CGA A; siPAEP10, GGA AGA GCC GUG CCG UUU UU; siPAEP11, CCA CGC UGC UCG AUA CUG AUU; and siPAEP12, ACA GCU GUG UUG AGA AGA AUU. The four duplex PAEP siRNAs, one siControl non-targeting pool and siGLO green transfection indicator were synthesized by Thermo Scientific Dharmacon (Lafayette, CO, USA).

siRNA knockdown. Transfection conditions were optimized by transfecting melanoma cells with siGLO of varying concentrations followed by analysis with fluorescence microscopy. For knockdown experiments, 624.38-Mel and MCC69B melanoma cells (1-2x10^5) were cultured in a 6-well plate in complete medium in a 5% CO_2 humidified atmosphere at 37˚C overnight. Gene-specific siRNAs or non-specific siControl were then transfected into tumor cells using DharmaFECT1 (Dharmacon, Lafayette, CO, USA) according to the manufacturer's instructions. After 8 h, the supernatant was removed and the cells were washed with phosphate-buffered saline (PBS) and maintained in complete medium for 24 h. The cells were incubated in serum-free RPMI-1640 medium. To assess the knockdown efficiency, transfected cells were collected for subsequent reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis, 48 and 72 h following transfection.

Construction of lentiviral vectors. To obtain stable PAEP shRNA melanoma cells, the lentiviral shRNA vector system was selected and three PAEP shRNAs (gene target sequences: shPAEP1, 5'-AAG ATC AAC TAT ACG GTG G-3'; shPAEP2, 5'-AAG AGC CGT GCC GTT TCT A-3'; shPAEP3, 5'-ATA AAC CCT TGG AGC ATG A-3') were screened. The lentiviral particles constructed by Thermo Scientific Dharmacon contained one TurboGFP (Evrogen, Moscow, Russia) reporter gene, one puromycin resistance gene and one of three PAEP shRNA sequences.

Optimization of polybrene concentration for enhanced transduction. Polybrene (hexadimethrine bromide) is a cationic polymer that increases the viral infection of mammalian cells by neutralizing the charge repulsion between the virus and cell surface. As it is cytotoxic to cells, the concentration of polybrene was optimized prior to lentiviral infection. Melanoma cells (624-Mel, 624.38-Mel and FEMX-I) were seeded into a 96-well plate at a density of 1x10^4 cells/well and cultured in a incubator in a 5% CO_2 atmosphere at 37˚C. Following overnight culture, the culture medium was replaced with fresh medium containing polybrene at different concentrations (0-10 μg/ml), and the cells were returned to the incubator for 24 h. Cell proliferation was determined using a cell proliferation kit (Pregene, Beijing, China) and the optical density (OD) of each well was determined at 490 nm with a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA). Assays were performed in triplicate at each concentration.

Determination of puromycin concentrations. The puromycin resistance gene in the lentiviral vector allows for the generation of stable cell lines by drug selection. Once the optimal concentration had been identified, transduced cells were selected and propagated. Melanoma cells (624-Mel, 624.38-Mel and FEMX-I) were seeded in a 96-well plate at a density of 1x10^4 cells/well and cultured in 5% CO_2 at 37˚C overnight. Puromycin (American Bioanalytical, Natick, MA, USA) was then added to each well at different concentrations, and the plate was returned to the incubator. Every 2 days, the cells were provided with fresh medium supplemented with puromycin of different concentrations. The OD of each well at different time-points was determined at 490 nm as previously described.

Multiplicity of infection (MOI) optimization of lentiviral particles. MOI, the ratio of lentiviral particles to cells, was optimized. Melanoma cells (624.38-Mel and FEMX-I) were seeded in a 96-well plate at a density of 1x10^4 cells/well and incubated in a 5% CO_2 atmosphere at 37˚C overnight. The cells were then infected with non-targeting shControl lentiviral particles at MOI ratios of 10, 50, 100 and 150 plaque-forming units (pfu)/cell in the presence of polybrene. After 48 h, the cells were observed using a Nikon Eclipse TE-2000U microscope (Nikon Instruments Inc., Melville, NY, USA) and collected for flow cytometric analysis.

Transient and stable transfection of melanoma cells with shRNA lentivirus. To assess the abilities of three PAEP shRNA lentiviral particles to silence PAEP gene expression, melanoma cells (624-Mel, 624.38-Mel and FEMX-I) were seeded in a 96-well plate at a density of 1x10^4 cells/well and were incubated in a 5% CO_2 atmosphere at 37˚C overnight. The cells were then infected with each of the three PAEP shRNA lentiviral particles at a MOI of 100 pfu/cell in the presence of polybrene. Shglyceraldehyde 3-phosphate dehydrogenase (GAPDH) and non-targeting shControl lentiviral particles were used as positive and negative controls, respectively. After 24 h, transduced cells were collected for qPCR analysis.

To obtain stable PAEP knockdown cell lines, melanoma cells were infected with a low MOI of lentiviral particles expressing PAEP shRNA. Melanoma cells (624-Mel, 624.38-Mel) were seeded in a 96-well plate at a density of 1x10^4 cells/well and were incubated in 5% CO_2 at 37˚C

overnight. The cells were then infected with PAEP shRNA3 lentiviral particles at a MOI of 10 pfu/cell in the presence of polybrene. After 24 h, the supernatant was replaced with complete medium supplemented with 1.5 μg/ml puromycin. Following puromycin screening and propagation, transduced cells were collected for qPCR and western blot analysis.

RT-PCR and qPCR. Total RNA from transduced cells was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration and purity were determined using a UV-2550 spectrophotometer (Shimadzu, Kyodo, Japan). Total RNA (5 μg) was reverse-transcribed into cDNA using a reverse transcription kit (Takara Biotechnology, Dalian, China). The resultant cDNA was amplified using the primers: Forward: 5'-AGG TTG GCA GGG ACC TGG CAC TC-3' and reverse: 5'-ACG GCA CGG CTC TTC CAT CTG TT-3' for PAEP; and forward: 5'-ACA CTG TGC CCA TCT ACG AGG-3' and reverse: 5'-AGG GGC CGG ACT CGT CAT ACT-3' for β-actin. PCR cycling conditions for PAEP were as follows: Initial denaturation at 95˚C for 1 min followed by 26 cycles of 95˚C for 10 sec, 60˚C for 15 sec, 72˚C for 45 sec and 72˚C for 7 min. PCR cycling conditions for β-actin were as follows: Initial denaturation at 94˚C for 2 min followed by 26 cycles of 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 30 sec and 72˚C for 7 min. The amplified products were analyzed using 1.5% agarose gel electrophoresis.

qPCR using gene-specific primers [unlabeled; PAEP-Hs00171462_m1 and GAPDH-Hs99999905_m1] and *Taq*Man MGB probes (6-FAM dye-labeled) purchased from Applied Biosystems (Foster City, CA, USA) was conducted in triplicate on a BioRad iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Transcript levels were normalized to GAPDH.

Western blot analysis. A total of 30 μg of each protein sample from the cell lysate or serum-free culture medium was loaded onto a sodium dodecyl sulfate-polyacrylamide gel and electrophoresed. Immunostaining was accomplished with the incubation of a PAEP antibody (2 μg/ml; Invitrogen, South San Francisco, CA, USA) followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research Labs, West Grove, PA, USA). Visualization was performed with enhanced chemiluminescence detection reagents (Thermo Fisher Scientific, Rockford, IL, USA) using a Fujifilm Luminescent Image Analyzer LAS-3000 (Fujifilm, Valhalla, NY, USA). The blot was analyzed using multi-gauge v3.1 software (Fujifilm).

Mass spectrometry. To observe changes in the secreted protein profiles of melanoma samples following PAEP-silencing, mass spectral analysis was conducted on a Thermo Fisher Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA, USA) with an Agilent 1200 Series Nanoflow LC (Agilent Technologies, Santa Clara, CA, USA) for sample introduction, as previously described (13-15). Prior to analysis, secreted proteins isolated from PAEP-knockdown and non-targeting knockdown 624.38-Mel cells were digested with trypsin in the presence of a reducing agent. Analysis data were then obtained at 60,000 resolution in the Orbitrap, while MS/MS spectra were obtained in parallel within the linear ion trap (LTQ). Analyses were performed in 3 h LC/MS runs and the data converted to search files and searched against the Mascot search engine (Matrix Science, available at www.matrixscience.com). The data were searched as a combined sample set (3 injection set) to produce the data shown. Mascot search data (Matrix Science, Boston, MA, USA) were automatically aligned between sets and the protein profiles were compared.

Statistical analysis. Statistical analysis was performed with the SAS statistical software (SAS Institute, Inc., Cary, NC, USA). Numerical data were expressed as the mean ± SD. Statistical differences between groups were assessed using analysis of variance and Dunnett's t-tests for each group. $P<0.05$ was considered to indicate a statistically significant difference.

Results

siRNA effectively silences PAEP gene expression. siRNA, as an inducer or RNAi, is a useful tool utilized to assess gene function. In the present study this technology was applied to knock down PAEP gene expression. Transfection conditions were optimized by transfecting melanoma cells with siGLO of varying concentrations followed by fluorescence microscopy and flow cytometric analysis. Transfection efficiencies in 624.38-Mel and MCC69B cells were 88.5 and 78%, respectively, at siGLO green and DharmaFECT1 concentrations of 100 nmol/l and 2 μl/ml, respectively (Fig. 1A). Four duplex siRNA sequences were analyzed to identify the sequence(s) that inhibited PAEP gene expression most efficiently in the melanoma cell lines. qPCR results showed that siRNAs 10, 11 and 12 were more effective than siRNA 9 in MCC69B and 624.38-Mel cell lines. Moreover, the mixture of PAEP-specific siRNA (siPAEP)-9-12 resulted in the greatest overall gene silencing of PAEP. Therefore, siRNAs 10, 11 and 12 were pooled to silence PAEP expression in subsequent experiments.

By utilizing the siPAEP10-12 pool to silence PAEP expression in MCC69B and 624.38-Mel cells, RT-PCR and qPCR results showed that PAEP mRNA was significantly downregulated (Fig. 2A and B; $P<0.05$, Dunnet's t-test). Moreover, the level of PAEP protein in the two cell lines decreased by 90% following siRNA transfection (Fig. 2C).

Lentiviral vector mediated shRNA temporarily silences PAEP gene expression. This study screened for and optimized the conditions of lentiviral vector-mediated shRNA transfection in the melanoma cell lines by testing various concentrations of polybrene and puromycin, the MOI of PAEP shRNA lentiviral particles and the ability of three shRNA molecules to effectively silence PAEP gene expression. MTS assay results demonstrated the optimal polybrene concentration to be 2 μg/ml (Fig. 3A). Similarly, a puromycin concentration of 1.5 μg/ml was selected as suitable to screen target cells in subsequent experiments (Fig. 3B and C). The melanoma cell lines were efficiently infected by lentiviral particles at a MOI of 100 pfu/cell (Fig. 1B).

As only a minority of constructs elicit efficient gene knockdown, studies typically prepare several candidate constructs. Thus, the present study screened three PAEP shRNA molecules to identify the most effective one. GAPDH and non-targeting shRNA were used as positive and negative

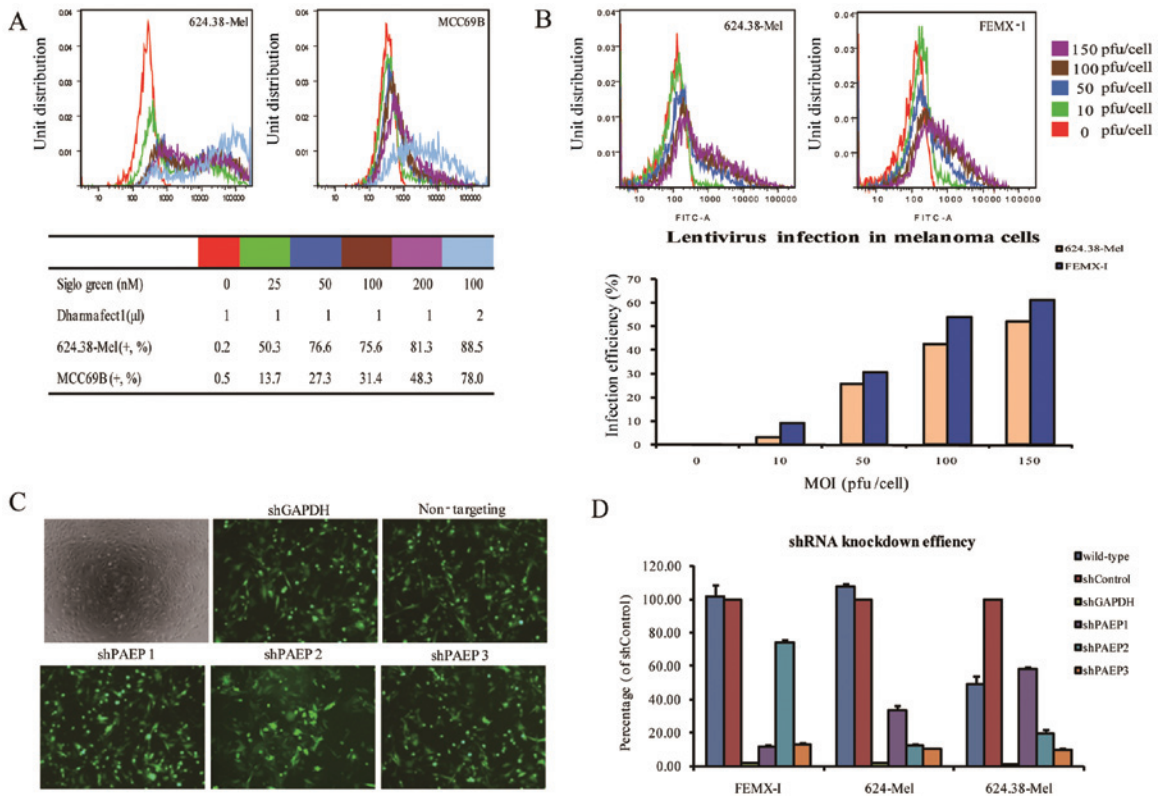

Figure 1. Optimization of siRNA and shRNA transfection conditions. (A) SiGLO green is used as an indicator to optimize transfection. Flow cytometric analysis shows that when the concentration of siGLO green was 100 nmol/l, the transfection efficiencies of 624.38-Mel and MCC69B cells were 88.5 and 78%, respectively. (B) The expression of GFP in 624.38-Mel and FEMX-I cells shown by the TurboGFP reporter following infection of the two cell lines at an MOI range of 0-150 pfu/cell. Flow cytometric analysis showed that the infection efficiencies for these two cell lines were ~50% at a MOI of 100. Infection efficiency increased when the MOI was increased. (C) Under a fluorescent microscope, 624.38-Mel cells were shown to be efficiently infected by all five vectors at a MOI of 100 (original magnification, x20). (D) Knockdown efficiency was detected by qPCR. Non-targeting shRNA served as a standard, and GAPDH was chosen as a positive control. Of the three lentiviral PAEP shRNAs tested, shPAEP3 was the most effective, silencing PAEP expression by 90%. GFP, green fluorescent protein; MOI, multiplicity of infection; PAEP, progestagen-associated endometrial protein; shRNA, small hairpin RNA; siRNA, small interfering RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

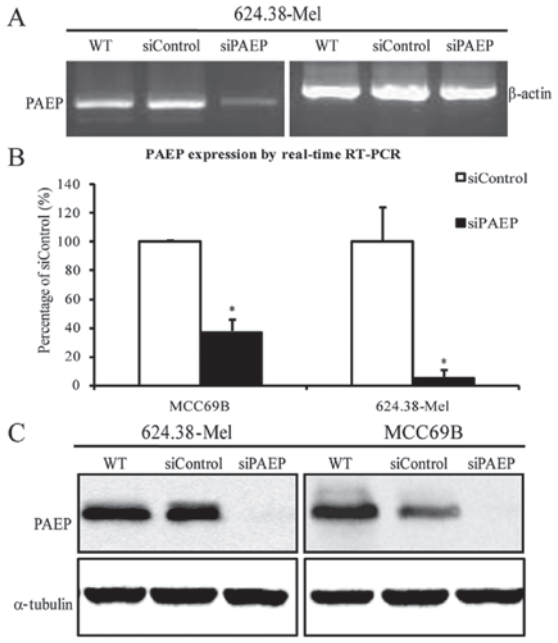

Figure 2. SiRNA inhibited PAEP expression. (A) PAEP mRNA level of 624.38-Mel cells following siPAEP10-12 transfection, determined by RT-PCR. (B) qPCR and (C) western blot analysis indicates that the secreted PAEP protein levels of two melanoma cell lines were decreased following siPAEP10-12 transfection. siRNA, small interfering RNA; WT, wild-type; PAEP, progestagen-associated endometrial protein.

controls, respectively. Utilizing siGLO as a visual indicator of successful transfection, fluorescent microscopy demonstrated that 624.38-Mel cells were efficiently infected by all five tested vectors at a MOI of 100 pfu/cell (Fig. 1C). Identical results were observed in 624-Mel and FEMX-I cells. Of the three lentiviral PAEP shRNAs tested, shPAEP3 exhibited the greatest ability to silence PAEP in each of the cell lines (90% expression decrease) (Fig. 1D). Therefore, shPAEP3 was selected to establish stable PAEP shRNA cell lines. Notably, the target site of shPAEP3 is near the poly A tail on the PAEP cDNA sequence.

Stable PAEP knockdown cell lines were successfully established. To obtain stable cell lines with a marked decrease of PAEP gene expression, 624.38-Mel and 624-Mel cells were infected with lentiviral particles at a low MOI (10 pfu/cell) and achieved ~3% infection efficiency (Fig. 4A). Following regular culture and puromycin screening, the cells and serum-free supernatant were collected for qPCR and western blot analysis. The results indicated that the expression of the PAEP gene in the shPAEP3 group was knocked down by 90% at the mRNA level and >80% at the protein level (Fig. 4B and C). Following puromycin screening and expansion in culture, two shPAEP3 and four shControl clones were derived from the 624.38-Mel and 624-Mel cells.

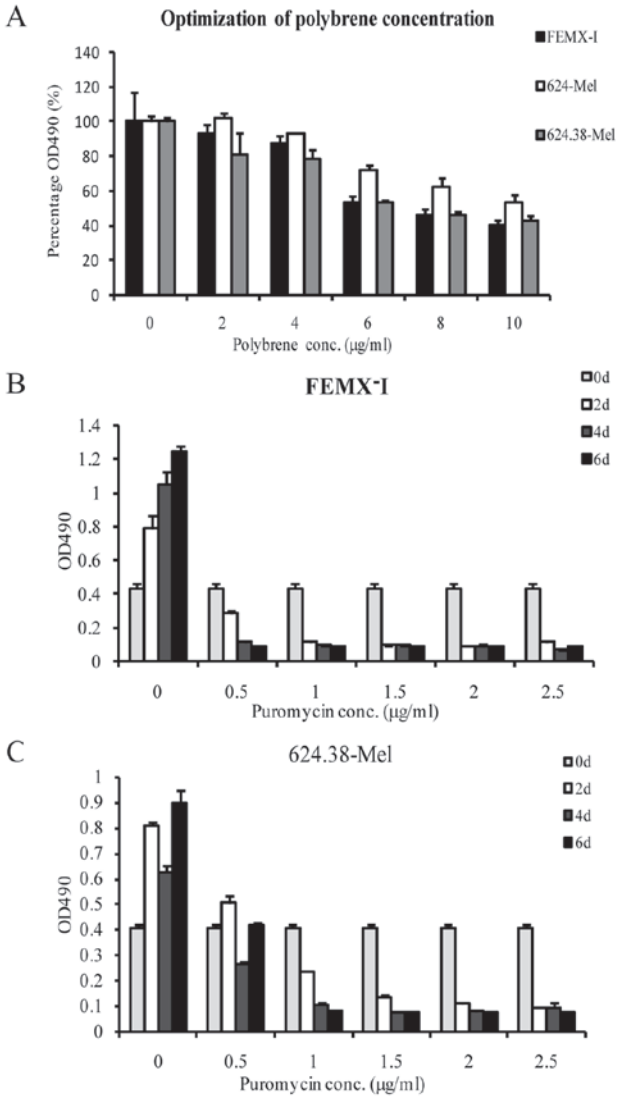

Figure 3. Optimization of polybrene and puromycin concentrations. (A) MTS assay for the proliferation of three melanoma cell lines with fresh medium containing a range of polybrene concentrations (0, 2, 4, 6, 8 and 10 μg/ml). A concentration of 2 μg/ml, polybrene exhibited the least toxicity to cell growth. (B and C) MTS assay was used to screen for the optimal concentration of puromycin, the lowest concentration that effectively eliminates all non-transduced cells within 4-6 days. A concentration of 1.5 μg/ml was selected as optimal.

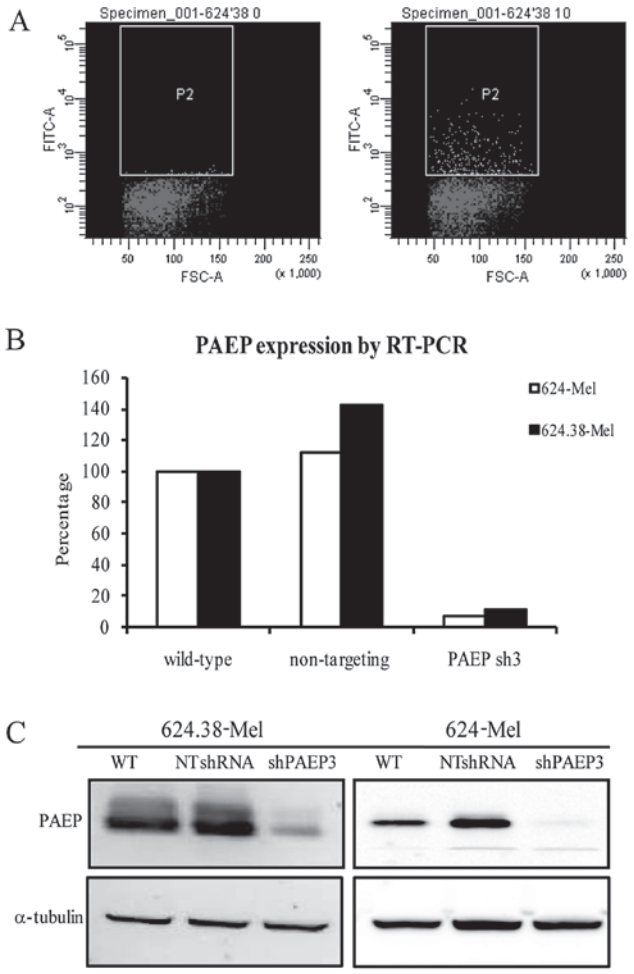

Figure 4. Establishment of stable melanoma cell lines stably expressing lentiviral shRNAs. (A) Approximately 3% of cells were infected by lentiviral particles at a low MOI. (B) In the shPAEP3 group, the expression of PAEP was knocked down by 90% at the mRNA level. (C) Stable transfection of 624.38-Mel and 624-Mel, together with their parental cells, was confirmed by western blot analysis. Protein samples were collected for PAEP from serum-free culture medium. WT, wild-type; NT shRNA, non-targeting small hairpin RNA; MOI, multiplicity of infection; PAEP, progestagen-associated endometrial protein.

PAEP knockdown results in alteration of certain secreted proteins. PAEP knockdown by shRNA resulted in an increase of certain secreted proteins and a decrease in others in the culture medium (Tables I and II). Elevated proteins included GDP dissociation inhibitor 2, transgelin-2 and thrombospondin-1. Decreased proteins included secreted phosphoprotein-1 (SPP-1), heat shock 90 kDa protein and laminin-α1.

Discussion

Melanoma is the most lethal form of skin cancer. When diagnosed early, melanoma may be cured by surgical resection. However, metastatic malignant melanoma is largely refractory to existing therapies and has a very poor prognosis, with a median survival rate of six months and a five-year survival rate of <5% (16).

Thus a large number of studies have been conducted with the aim of identifying a correlation between gene alteration and tumor development. Our previous study identified a list of specific genes that were overexpressed and underexpressed throughout the various time-points of melanoma tumor progression (17). PAEP is one of the genes that was demonstrated to exhibit a higher level of expression in freshly procured melanoma tissues and daughter cell lines (7). The gene has also been demonstrated to be differentially involved in certain tumor (18,19). Several studies have suggested that PAEP inhibits tumor cell growth and promotes cell differentiation as a tumor suppressive factor; however, other authors have demonstrated that PAEP increases the invasion of endometrial adenocarcinoma cells (20) and melanoma (10). Therefore, the function of PAEP gene expression in tumorigenesis and tumor progression remains unclear. The silencing of intact wild-type gene expression, a valuable method of functional discovery, is required to be utilized to characterize PAEP in cancer systems.

Table I. Protein expression upregulated by PAEP shRNA in 624.38-Mel cells.

Abbreviation	Protein	Fold change[a]	Involvement in cancer
GDI-2	GDP dissociation inhibitor-2	16.84	Inhibits tumor cell invasion and metastasis in stomach cancer
Tagln-2	Transgelin-2	nd[b]	Tumor supression factor in ovarian cancer
MMP-2	Matrix metalloproteinase-2	6.58	Unknown
TSP-1	Thrombospondin-1	5.05	Induces endothelial cell apoptosis, thus inhibits tumor angiogenesis

[a]Fold change compared to control; [b]protein was not detected in control group. shRNA, small hairpin RNA; nd, not detected; PAEP, progestagen-associated endometrial protein.

Table II. Protein expression downregulated by PAEP shRNA in 624.38-Mel cells.

Abbreviation	Protein	Fold change[a]	Involvement in cancer
SPP-1	Secreted phosphoprotein-1	nd[b]	Promotes tumor cell migration and inhibits cell apoptosis
LN-α1	Laminin-α1	nd[b]	Activates tumor migration
HSP-90	Heat shock 90 kDa protein	2.38	Inhibits cell apoptosis
Vgf	VGF nerve growth factor	2.04	Unknown

[a]Fold change compared to control; [b]protein was not detected in PAEP-knockdown group. shRNA, small hairpin RNA; nd, not detected; PAEP, progestagen-associated endometrial protein.

Since the identification of RNAi in *Caenorhabditis elegans* in 1998, this mechanism has been demonstrated to be conserved in a wide variety of species, including insects, plants and mammals. siRNA, an important inducer of RNAi, is thus a useful tool to assess gene function. Formation of siRNA occurs when dicer binds to dsRNA and digests it into duplexes of 21-23 nucleotides. These, in turn, are incorporated into the RNA-induced silencing complex, which has been suggested to eliminate one of the strands and thus initiate a cyclical process as the siRNA associates with novel target molecules (21,22). In the present study, this technique was used to silence PAEP expression. Four duplex siRNAs were selected using the PAEP mRNA sequence and significantly suppressed PAEP expression by as much as 80% at the post-transcriptional and translational levels.

As siRNA only binds to its respective mRNA counterpart in the cytoplasm, its inhibitory effect is transient. Alternatively, there is another method by which to prolong inhibition. In mammals, shRNA may be expressed by using several expression vectors. Studies have demonstrated that adenovirus and lentivirus vectors are useful tools for delivering a target gene into the nucleus. However, adenoviral-mediated gene expression is not maintained for long, and adenovirus vectors may induce an immune response in the host (23-25). By contrast, the lentiviral vector has several useful characteristics for RNAi experiments, including broad host tropism and stable gene transduction to dividing and non-dividing cells, permitting stable depletion of target genes (26). Accordingly, a lentiviral vector system was used to deliver and express PAEP shRNA in the melanoma cells in order to establish stable cell lines. Following optimization of the transfection methods, a stable PAEP shRNA cell line with low MOI was successfully established. qPCR confirmed that PAEP gene expression in shPAEP stable transfectants was reduced by >80%, and western blot analysis validated these results by showing a reduction in PAEP protein of >75%.

Moreover, it was demonstrated that knockdown by PAEP-specific shRNA resulted in the alteration of the secreted protein profiles in the melanoma cell lines, as determined by protein changes in the culture medium. TSP-1, one of the proteins highly secreted upon PAEP-silencing, is known to induce endothelial cell apoptosis, thus inhibiting tumor angiogenesis, and is considered to be a tumor suppressive factor (27-29). It is therefore possible that PAEP promotes tumor cell growth at least in part by downregulating TSP-1 expression. The presence of SPP-1, a putative tumor oncogene (30-32), in the medium was lost following the knockdown of PAEP. Thus, secretion of SPP-1 is directly related to the presence of PAEP. Our previous study demonstrated that silencing the PAEP gene expression decreased the migration and invasion of melanoma cells and inhibited tumor growth in a human xenograft model (10). These results suggested that PAEP may act as a tumor-promoting factor influencing the biological behavior of melonom and knockdown of PAEP gene was essential for this observation. PAEP is regarded as an immunosuppressive factor in embryonic implantation, and may hamper immunological responses in the tumor microenvironment. In our subsequent study, the PAEP-silenced cell

models established in the present study may be used to further investigate this hypothesis. Considering the significance of silencing the PAEP gene, this study thoroughly demonstrated and characterized the procedure of gene knockdown and may be used for other gene function studies in cancer systems.

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