

Functional characterization of the progesterone-associated endometrial protein gene in human melanoma

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Abstract

Utilizing gene microarray profiling of melanoma samples, we have recently identified a novel gene overexpressed in both thick primary and metastatic melanomas. This gene, progesterone-associated endometrial protein (PAEP), has never before been implicated in the oncogenic processes of melanoma, with its true function in oncogenesis and tumour progression relatively unknown. Overexpression of the PAEP gene in freshly procured thick primary and metastatic melanoma samples (58%) and daughter cell lines (77%) is confirmed by quantitative RT-PCR, immunohistochemistry, Western blotting and mass spectrometric analysis. We suggest that PAEP gene overexpression is involved with melanoma tumour progression as well as an aggressive phenotype. Transfection of melanoma cells with PAEP small interfering RNA (siRNA) reveals a significant decrease in soft agar colony formation and a marked inhibition of both cell migration and cell invasion. Furthermore, we establish stable melanoma transfectants *via* PAEP lentiviral small hairpin RNA (shRNA), examine their growth characteristics in a murine xenograft model and reveal that tumour growth is significantly inhibited in two separate melanoma cell lines. Our data strongly implicate the PAEP gene as a tumour growth promoter with oncogenic properties and a potential therapeutic target for patients with advanced melanoma.

Keywords: melanoma • progesterone-associated endometrial protein • oncogene • RNA interference

Introduction

Progesterone-associated endometrial protein (PAEP, pregnancy-associated endometrial $\alpha 2$ globulin, placental protein 14 and glycodelin) is a 28-kD glycoprotein of the lipocalin superfamily. This protein is synthesized by cells of the human reproductive tract, especially the secretory endometrium and deciduas of early pregnancy. Others have found a remarkably high PAEP concentration within amniotic fluid during the first half of pregnancy [1, 2]. PAEP has also been identified within cells of the

megakaryocytic lineage, including platelets [3]. Lastly, PAEP has been previously suggested to contribute to the maternal immunotolerance involved in embryogenesis and human foetal development [4–8]. Although the definitive role of PAEP in malignancy is relatively unknown, it is well described as having a negative influence upon the human immune system, with an overall immunosuppressive effect.

Recently, it has been shown that PAEP is frequently expressed in tumours derived from various reproductive organs and gynecologic malignancies, such as endometrial, ovarian, breast and cervical cancers [9–12]. However, we still know relatively little about the true function and role of PAEP gene expression in tumorigenesis and tumour progression. The utilization of histone deacetylase inhibitors (HDACI) has been shown to stimulate cell migration in human endometrial adenocarcinoma *via* the up-regulation and overexpression of PAEP [13]. Song *et al.* revealed that increased

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migration and tube formation of human umbilical vein endothelial cells (HUVECs) occurred in the presence of a synthesized PAEP peptide or PAEP-rich amniotic fluid, mediated by vascular endothelial growth factor (VEGF), suggesting that PAEP is intimately involved in neovascularization during tumour growth [14]. Here, we show that PAEP highly expresses in both thick primary and metastatic melanoma tissues and daughter cells, subsequently promoting tumour growth in human melanoma.

Melanomagenesis is a result of the malignant transformation of neural crest-derived melanocytes [15]. It is one of the most aggressive forms of human cancer and is responsible for 6/7 skin cancer-related deaths, with only rare long-term survivors once metastatic disease has developed [16]. Thus, we wanted to gain an improved understanding of the metastatic process in melanoma, specifically focusing on the genes involved in this complex process. In this study, we observed a high level of PAEP gene expression in thick primary and metastatic melanoma samples as well as in daughter cell lines. We also show that PAEP overexpression in human melanoma is involved with both proliferative and migratory potential, with its inhibition of gene expression resulting in delayed tumour growth in human melanoma.

Materials and methods

Tumour specimens and cell lines

Nineteen tumour specimens, comprised of seven thick primary cutaneous melanoma samples (>4 mm in Breslow's depth) and 12 metastatic melanoma samples (lymph node, subcutaneous, adrenal and brain), were surgically excised from patients under an Investigational Review Board (IRB) approved tissue procurement protocol (IRB#101751, MCC13448), and were cryopreserved in liquid nitrogen within 10 min. of removal. Additionally, we included two normal human epidermal melanocyte (NHEM) cell lines (NHEM2751 and NHEM721) and two normal human skin samples for comparative controls.

Melanoma daughter cell lines derived from freshly procured tumour samples were established utilizing previously published techniques [17, 18] and serially passed in culture less than 15 times in all cases. We utilized the following cell lines originally procured from melanoma patients: two thick primary melanomas (MCC13 and MCC80A), three lymph node metastases (MCC67, MCC74 and MCC80B) and five distant metastases (MCC12A, MCC12F, MCC69A, MCC69B and MCC81). We also utilized three metastatic melanoma cell lines obtained from the National Cancer Institute, Surgery Branch: 624-Mel, 624.38-Mel and A375. All melanoma cell lines were cultured in RPMI-1640 culture media supplemented with 10% FBS. NHEM cells were obtained from ATCC and cultured according to the manufacturer instructions (Cambrex, Walkersville, MD, USA).

ShRNA stable transfectants were screened and subsequently maintained in RPMI-1640 culture media supplemented with 10% FBS and 1.5 µg/ml Puromycin (American Bioanalytical, Natick, MA, USA). Stable transfectants were derived from cell cultures with less than 10 passages in order to minimize the possible impact of clonal diversification and phenotypic instability *in vitro*.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA of cryopreserved tissues and cultured cell lines was isolated using TRI Reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. Gene-specific primers (unlabelled) and TaqMan[®] MGB probes (6-FAM[™] dye-labelled) were purchased from Applied Biosystems (Foster City, CA, USA): PAEP-Hs00171462_m1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-Hs99999905_m1 and cyclophilin B (CYPB)-Hs00168719_m1. The relative amounts of each transcript of the tested genes were normalized to GAPDH or CYPB when GAPDH was applied as a positive control. Real-time qRT-PCR was carried out in triplicate and run on a BioRad IQ5 multicolor real-time PCR detection System (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

The mouse monoclonal PAEP antibody (001-16-1, Santa Cruz, CA, USA) applied for immunohistochemistry was raised against human PAEP purified from second trimester amniotic fluid. Formalin-fixed and paraffin-embedded melanoma tissue histology sections were incubated with PAEP antibody after pretreatment of the sections in citrate buffer (pH 6.0) at 95°C for 20 min. then room temperature for 20 min. The staining was developed with a Dako Autostainer using a peroxidase-based visualization kit (Dako, Glostrup, Denmark) with a diaminobenzidine substrate chromogen system (Dako). For a negative control, we replaced the primary antibody with PBS.

Western blotting

Thirty µg of each protein sample from cell lysate or serum-free culture medium was loaded within an SDS-PAGE gel followed by electrophoresis. Immunostaining was performed with PAEP (Invitrogen, South San Francisco, CA, USA), Phospho-eIF2α (Cell signaling, Danvers, MA, USA) or α-tubulin (Cell signaling) antibody followed by secondary antibody conjugated to horseradish peroxidase as described previously [19]. The blot was visualized with a Fujifilm Luminescent image analyser LAS-3000 and analysed utilizing Multi-Gauge V3.1 software (Fujifilm, Valhalla, NY, USA). The target bands of PAEP, Phospho-eIF2α and α-tubulin were visualized at 30, 38 and 52 kD, respectively.

Mass spectrometry

The analysis of the secreted proteome was conducted as previously described [20, 21]. Briefly, the isolated secreted proteins were digested with trypsin in the presence of a reducing agent, followed by analysis of each digest on a ThermoFisher Orbitrap mass spectrometer (ThermoFisher, Waltham, MA, USA) with an Agilent nano-1200 LC for sample introduction. Mass Spectrometry analysis data were acquired at 60,000 resolution in the Orbitrap, while MS/MS spectra were acquired in parallel within the linear ion trap (LTQ). All analyses were performed in triplicate 180 min. LC/MS runs and the data converted to search files and searched against the Mascot search engine (www.matrixscience.com). Each analysis of the triplicate was compared for consistency and then the data searched as a combined sample set (three injection set) to produce the data reported. Mascot search data (Matrix) were automatically aligned between sets and the protein profiles compared. Repeat secretome preparations of the melanoma cell lines revealed almost identical results.

Small interfering RNA (siRNA) knockdown

One siGLO green transfection indicator, one siControl non-targeting pool and four duplex PAEP siRNAs (target sequences: siPAEP9-UCAACUUAUCGGUGGCGAAUU; siPAEP10-GGAAGAGCCGUGCCGUUUUU; siPAEP11-CCAGCUGCUGCAUACUGAUU; and siPAEP12-ACAGCUGUGU-UGAGAAGAAUU) were synthesized by ThermoFisher Dharmacon (Lafayette, CO, USA). Transfection conditions were optimized by transfecting melanoma cells with siGLO of varying concentrations followed by flow cytometric analysis. Briefly, melanoma cells ($1-2 \times 10^5$) were cultured in a 12-well plate in RPMI-1640 culture media supplemented with 10% FBS. PAEP siRNAs or nonspecific siControl were transfected into tumour cells using DharmaFECT1 (Dharmacon) according to the manufacturer's instructions. To assess the knockdown efficiency, transfected cells were collected for subsequent real-time qRT-PCR and Western blot analysis at 48 hrs and 72 hrs after transfection, respectively.

Colony formation in soft agar

Melanoma cells were transfected with 100 nM siPAEP10-12 or siControl. We incubated all cells for 48 hrs and re-suspended an aliquot corresponding to 500 cells in 75 μ l of 0.3% agar media (agar in RPMI1640 containing 10% FBS). The cell mixture was then layered on 50 μ l of 0.5% bottom agar media in a 96-well plate. Each sample was assayed in triplicate, followed by a colorimetric MTT assay carried out after an 8-day incubation period using an *in vitro* tumour sensitivity assay kit (Cell Biolabs, San Diego, CA, USA).

Scratch assay

Monolayer melanoma cells at 80–90% confluence were transfected with or without siPAEP10–12 or siControl, incubated for 24 hrs, and scratched by a 1 ml pipette tip (0 hr). At 0 hr and 18 hrs, the scratched cultures were photographed and visually compared for differences in cell migration utilizing an inverted microscope Zeiss Axiovert 200M (Carl Zeiss, Jena, Germany).

Transwell migration assay

Melanoma cells with or without transfection of siPAEP10-12 or siControl were suspended in complete culture medium at 2×10^5 cells/ml. Each 100 μ l of cells was then applied onto the upper migration chambers of the transwell plate (6.5 mm, 8.0 μ m pore size, Corning, Acton, MA, USA) and allowed to migrate for 6 hrs. The cells were fixed with 2% paraformaldehyde and then stained by 0.5% crystal violet. Utilizing standard light microscopy, migrating cells were counted in 10 randomly chosen fields, with the relative migration ratio of wild-type cells set as 100%.

Matrigel invasion assay

The lower chambers of the 24-well Matrigel invasion plate (8.0 μ m pore size, BD, Bedford, MA, USA) were filled with 0.75 ml RPMI-1640 containing 5% FBS and 6.5 μ g/ml human fibronectin (BD) as a chemoattractant. 5×10^4 tumour cells in 0.5 ml serum-free RPMI1640 were added to the

upper chambers and the plate incubated at 37°C in 5% CO₂ humidified atmosphere for 24 or 48 hrs. Further staining and recording of results was performed in a similar fashion to the methods utilized for the transwell migration assay.

Small hairpin RNA (shRNA) lentivirus infection

For *in vivo* experiments, we established the PAEP shRNA stable melanoma cells after infection with PAEP shRNA lentivirus. Three PAEP shRNA lentiviral particles (gene target sequences: shPAEP1 5'-AAGATCAACTATACG-GTGG-3'; shPAEP2 5'-AAGAGCCGTGCCGTTTCTA-3'; shPAEP3 5'-ATAAAC-CCTTGGAGCATGA-3') were screened and the optimal shRNA was chosen which exhibited the greatest degree of PAEP gene silencing. Briefly, melanoma cells were infected with each of three PAEP shRNA lentiviral particles at a MOI (multiplicity of infection, the ratio of lentiviral particles to cells) of 100 TU (transducing units, the number of viral particles in a solution that are capable of transducing and integrating into cells) per cell in the presence of 2 μ g/ml of Polybrene. shGAPDH and shControl non-targeting shRNA lentiviral particles were applied as positive and negative controls, respectively. The cells were then incubated for 48 hrs and analysed by confirmatory real-time qRT-PCR analysis.

To confirm that the melanoma cells possessed stable transfection of the shPAEP lentiviral constructs, we infected cells at low MOI of 5 TU/cell and subsequently selected for puromycin resistance after 48 hrs of shPAEP3 infection. For all stable transfectants, PAEP knockdown efficiency was confirmed by real-time qRT-PCR and Western blotting analysis for mRNA and protein expression, respectively.

Human melanoma xenograft model in athymic mice

We utilized a murine model with human melanoma xenografts as previously described [22]. In brief, 2.0×10^6 cells were suspended in 100 μ L of Hank's Balanced Salt Solution and injected subcutaneously (s.c.) in the flank of 4–6 week old, female athymic mice (Harlan Sprague-Dawley, Indianapolis, IN, USA). We utilized six groups of mice for the MCC69B cell line: 2 shPAEP3s, 1 wild-type, 1 empty vector and 2 shControl clones, and five groups for 624.38-Mel: 3 shPAEP3s, 1 empty vector and 1 shControl clones. All groups comprised 8 mice, with tumour measurements performed weekly and the mean tumour diameter calculated by taking the square root of the product of orthogonal measurements. Mice were sacrificed after 4–5 weeks, reached 10 mm in mean diameter or caused ulceration of the overlying skin. All animals were maintained and cared for under the guidelines for animal research according to the National Institutes of Health and the University of South Alabama. All experiments were performed with the approval of the IACUC committee at the University of South Alabama.

Statistical analysis

Statistical differences between groups were assessed using ANOVA analysis and DUNNETT t-tests for each group, utilizing SAS statistical software (SAS, Cary, NC, USA). The minimal level of significance was set at a value of $P < 0.05$ or 0.01, with all values presented as the mean \pm standard deviation (SD), or the mean \pm standard error of the mean (SEM) for tumour diameter.

presence of four non-overlapping sequences and a pair of overlapping c-terminal sequences shown in bold in Figure 1E. Since glycosylation prevents mass spectrometry sequencing, we did not detect the sequence 79–93 due to the glycosylated Asn81 [25, 26]. However, we still detected a weak peak for the sequence 33–58 although it is reported to be glycosylated at Asn46 [25, 26], indicating a small amount of unglycosylated peptide was present. The sequence 102–142 contains a predicted glycosylation site [27] reported not to be occupied [25, 26]; however, the peptide was too big to be sequenced (4.7 kD). Thus, a good coverage of the protein was obtained to provide positive proof of its presence. Therefore, our data strongly suggest that PAEP is a secreted protein in melanoma cells, with only trace amounts retained intracellularly.

SiRNA silencing of PAEP gene expression results in the inhibition of melanoma colony formation in soft agar

We wanted to further examine the functional role of PAEP overexpression in melanoma and thus utilized RNA interference methods to assess gene function [28, 29]. We analysed four duplex siRNA sequences in order to find the sequence(s) that inhibit PAEP gene expression most efficiently in melanoma cells. Real-time qRT-PCR results show that for three melanoma cell lines (MCC69B, 624-Mel and 624.38-Mel), the combination of siPAEP10, 11 and 12 was more effective when compared to each of four siPAEPs alone. Additionally, the mixture of siPAEP10–12 resulted in the best overall gene silencing of PAEP, with a silencing efficiency of ~70–80% (Fig. 2A). Western blot analysis of all cell lines transfected with the siPAEP10–12 confirmed the marked inhibition of PAEP gene expression ranging from 82% to 97% (Fig. 2B).

Therefore, we utilized the siPAEP10–12 pool to silence PAEP expression in MCC69B and 624.38-Mel cells. This was followed by culturing the melanoma cells in soft agar to examine whether the inhibition of PAEP gene expression affected cell colony formation. This revealed a significant decrease in melanoma cell colony formation in the two cell lines (Fig. 2C left). Subsequent analysis of each cell line with a colorimetric MTT assay revealed significant decreases of 46% and 27%, respectively, in total cell numbers in PAEP siRNA-treated MCC69B and 624.38-Mel samples ($P < 0.05$, Fig. 2C right).

Silencing of PAEP gene expression decreases the migration and invasion of melanoma cells

Scratch assays were performed to examine the effect of PAEP expression on collective cell migration. Confluent monolayer cells, MCC69B, 624-Mel and 624.38-Mel, transfected with or without siPAEP10-12 or siControl non-targeting pool, were scratched, cultured for 18 hrs and then photographed with a phase contrast microscope to capture the wound area (Fig. 3A). For all three

melanoma cell lines, the collective cell migration was significantly decreased after PAEP gene silencing, while cells transfected with siControl were similar to the wild-type groups.

We next investigated the influence of PAEP gene expression on single melanoma cell migration utilizing a transwell chamber assay. As shown in Figure 3B, for all three cell lines, MCC69B, 624-Mel and 624.38-Mel, cell migration was significantly suppressed by 25–50% after PAEP siPAEP10–12 transfection compared to wild-type and non-targeting controls ($P < 0.05$).

We then further examined the affect of PAEP gene expression upon the invasive capacity of melanoma cells. The transfected 624.38-Mel cells were placed into invasion chambers coated with a Matrigel basement membrane matrix, with fibronectin added to the lower chamber serving as a chemoattractant to melanoma cells. After incubating for 24 hrs, we observed both wild-type and siControl transfected cell invasion through the matrix. However, the invasion of siPAEP10-12 transfected cells was markedly inhibited by ~68% compared to wild-type controls. We observed similar results after 48 hrs of incubation. The invasion ratio of siPAEP10-12 group was 26% relative to that of the wild-type control (Fig. 3C, left). Statistical analysis revealed that there were significant differences between invasive capacities when comparing PAEP siRNA to control groups ($P < 0.05$, Fig. 3C right).

PAEP expression is transcriptionally silenced by lentiviral shRNA

We established stable lentiviral shRNA cell transfectants with the PAEP gene in order to better understand the role of PAEP in progressive tumour growth *in vivo*. Applying the built-in reporter gene, TurboGFP, we successfully transfected the lentiviral vector into human melanoma cells (data not shown), providing the delivery vehicle for PAEP shRNA. Three PAEP shRNA lentiviral vectors were screened for the optimal knockdown efficiency of PAEP gene expression. Applying the shGAPDH and shControl as positive and negative controls, respectively, we utilized qPCR to show that shPAEP1, shPAEP2 and shPAEP3 reduced PAEP mRNA expression by 54.5 ± 12.6 , 84.3 ± 3.5 and $88.6 \pm 2.0\%$ (mean \pm SD) in three melanoma cell lines (MCC69B, 624-Mel and 624.38-Mel), respectively. We therefore utilized shPAEP3 as the most effective shRNA.

Five MCC69B stable transfectants (1 empty vector, 2 shControl and 2 shPAEP3 clones) and five 624.38 stable transfectants (1 empty vector, 1 shControl and 3 shPAEP3 clones) were established by lentiviral vector infection at low MOIs (5 TU/cell), followed by Western blotting and real-time qRT-PCR validation. In the PAEP3 group, we show an overall knockdown efficiency of over 90% at the mRNA level and 75–99% at the protein level (Fig. 4A and B). To monitor whether the shRNA constructs were possibly exerting effects upon the activation of interferon and downstream effectors [30, 31], expression of Phospho-eIF2 α (P-eIF2- α) was also examined. We did not observe any significant differences in gene expression for P-eIF2- α (Fig. 4B). Thus, we

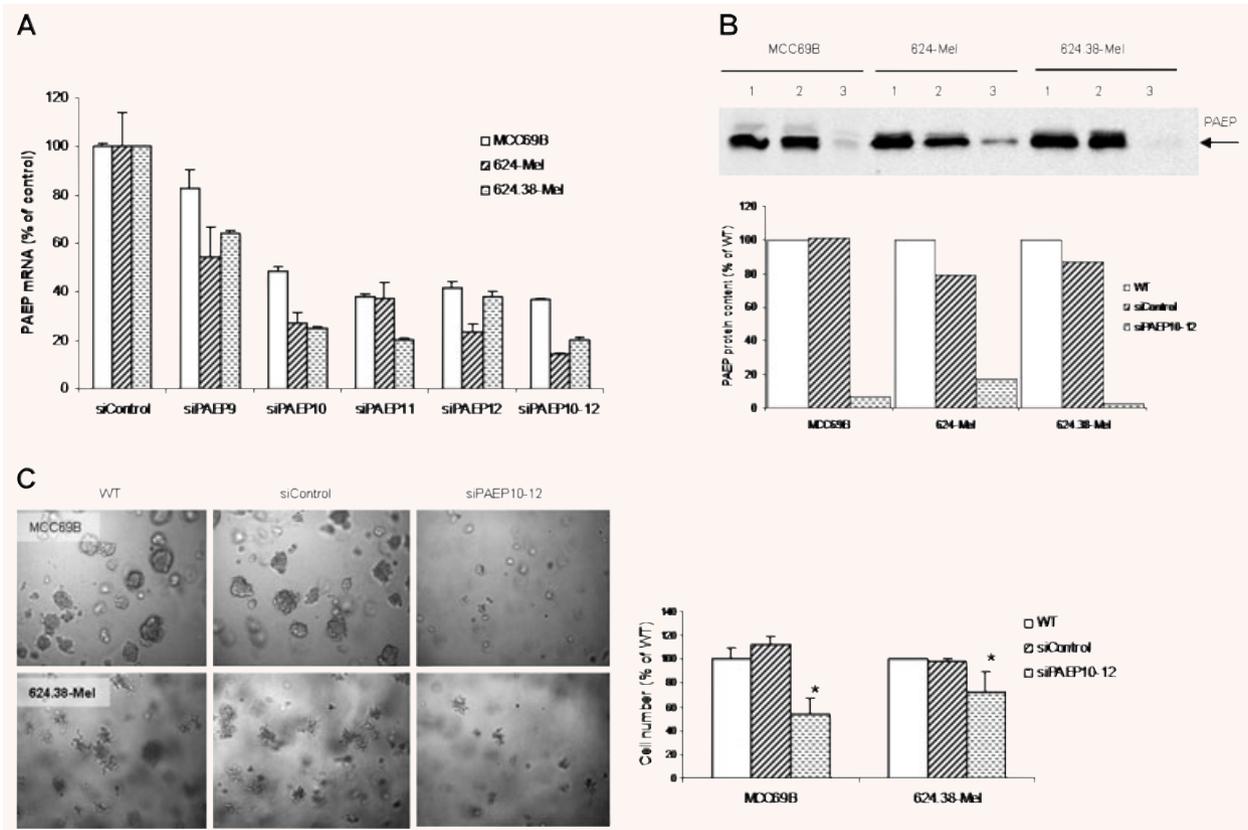


Fig. 2 siRNA knocks down PAEP expression and decreases the colony formation of melanoma cells. **(A)** Real-time qRT-PCR shows the PAEP mRNA levels of three melanoma cell lines are significantly decreased after siPAEP10, 11 or 12 transfection, with the mixture of three duplexes delivering the best silencing by 60–80%. **(B)** Western blot (upper) and Multi Gauge V3.1 software analysis (lower) indicates the secreted PAEP protein level of three melanoma cell lines is decreased by >80% after siPAEP10–12 transfection. 1, Wild-type (WT); 2, siControl; 3, siPAEP10–12. **(C)** Melanoma cells, MCC69B and 624.38-Mel, transfected with or without siPAEP10–12 and siControl. Left: soft agar culture revealed a significant decrease in colony formation by siPAEP10–12 transfection into melanoma cells (10× magnification). Right: colorimetric MTT assay revealed a significant decrease in total cell numbers in siPAEP10–12 treated MCC69B and 624.38-Mel samples. * DUNNETT t-test, $P < 0.05$.

show that the PAEP gene can be effectively silenced at the post-transcriptional and protein level utilizing the stable transfection of shPAEP3 into melanoma cells.

Silencing of PAEP expression inhibits tumour growth in a human xenograft model

Our *in vitro* results thus far support a central role for PAEP in contributing to melanoma cell growth, migration and invasion. Thus, we wished to further analyse the *in vivo* effects of the PAEP gene utilizing the shPAEPs clones in a human xenograft tumour model. To this end, we injected shPAEP stable melanoma cell transfectants subcutaneously into athymic mice. In MCC69B cells, 3/8 (shPAEP3-1) and 4/8 (shPAEP3-2) of the mice injected with the shPAEP3 transfectants developed tumours. Of the tumours that did form, growth initiation was delayed by approximately 2 weeks

with a concomitant marked attenuation in tumour growth. In contrast, all mice injected with wild-type, empty vector or shControl cells developed progressively enlarging tumours (Fig. 5A). The difference in overall tumour size between shPAEP3 and control groups was found to be statistically significant (mean ± SEM: 0.6 ± 0.3 mm, 1.2 ± 0.5 mm, 6.7 ± 0.4 mm, 6.2 ± 0.2 mm, 6.3 ± 0.4 mm, and 6.1 ± 0.2 mm for shPAEP3-1, shPAEP3-2, wild-type, empty vector, shControl-1 and shControl-2 groups, respectively. $P < 0.01$, DUNNETT t-test). Tumour growth was inhibited by 86% in the group of mice injected with stable shPAEP3 transfectants (Fig. 5B). In 624.38-Mel cells, the mice in all five groups developed tumours. However, similar to the MCC69B groups, the tumour growth of three shPAEPs clones was significantly decreased by 33% compared to empty vector and shControl cells ($P < 0.01$, DUNNETT t-test, Fig. 5C). To further confirm the specificity of shPAEP knockdown, we also applied a shPAEP2 clone derived from MCC69B cells, which reduced PAEP

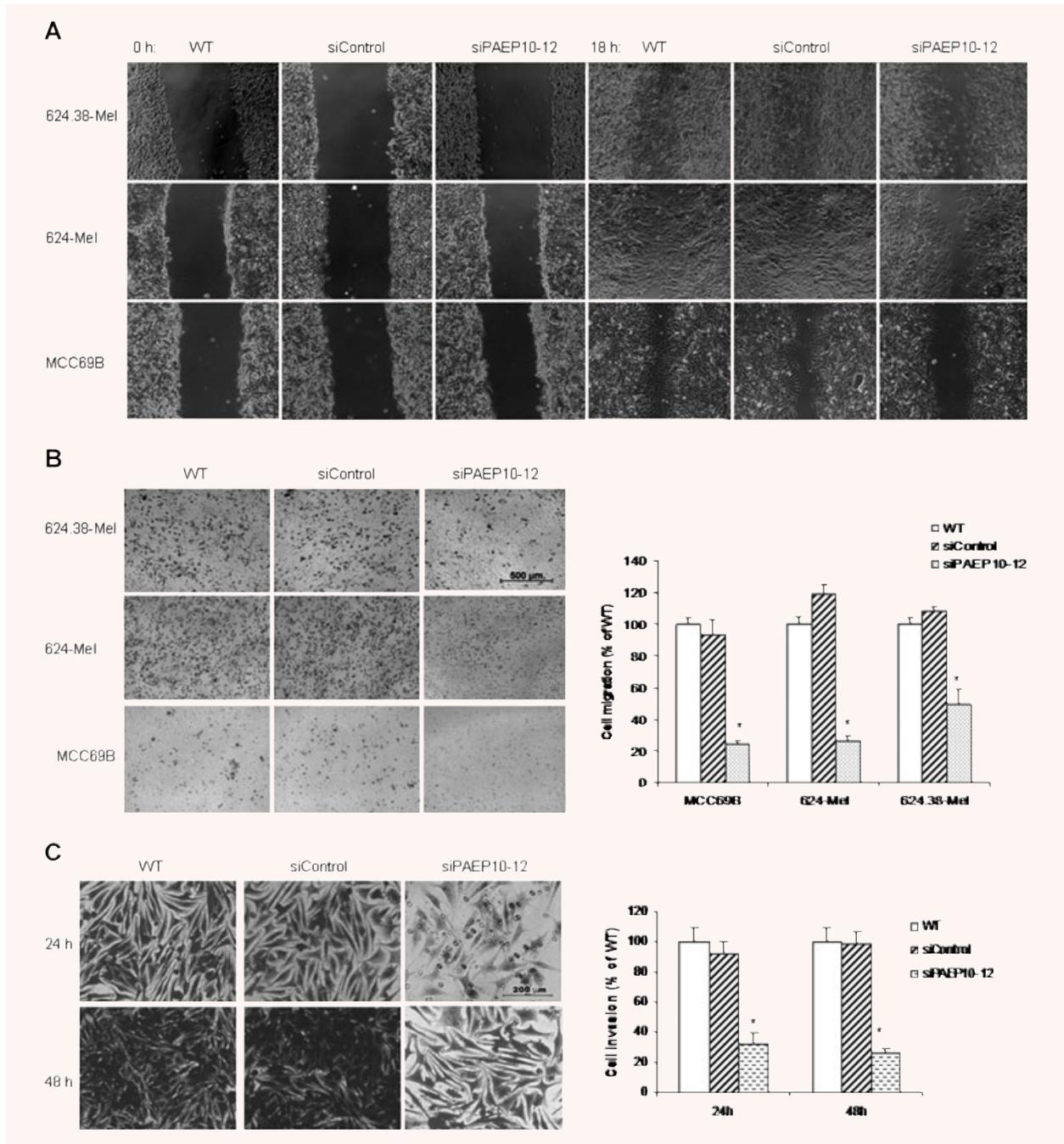


Fig. 3 The blockage of PAEP expression decreases the migration and invasion of melanoma cells. **(A)** Melanoma cells were transfected with or without siPAEP10–12 and siControl and scratched followed by 24 hrs incubation. The scratched cultures were photographed under an inverted microscope. **(B)** Melanoma cells with or without transfection of siPAEP10–12 or siControl were plated onto the upper transwell chambers and allowed to migrate for 6 hrs. Cells migrated to the underside of the chamber were counted in 10 randomly chosen fields under microscope (left; 10 \times magnification) and were analysed by DUNNETT t-test (right). Relative migrated cells of parental cells set at 100%. * $P < 0.05$. **(C)** Melanoma cells with or without transfection of siPAEP10–12 or siControl were added to the upper chambers of Matrigel invasion chambers and were incubated for 24 hrs or 48 hrs. Further observation (left; 40 \times magnification) and analysis (right) were performed as mentioned above for the migration assay. * $P < 0.05$.

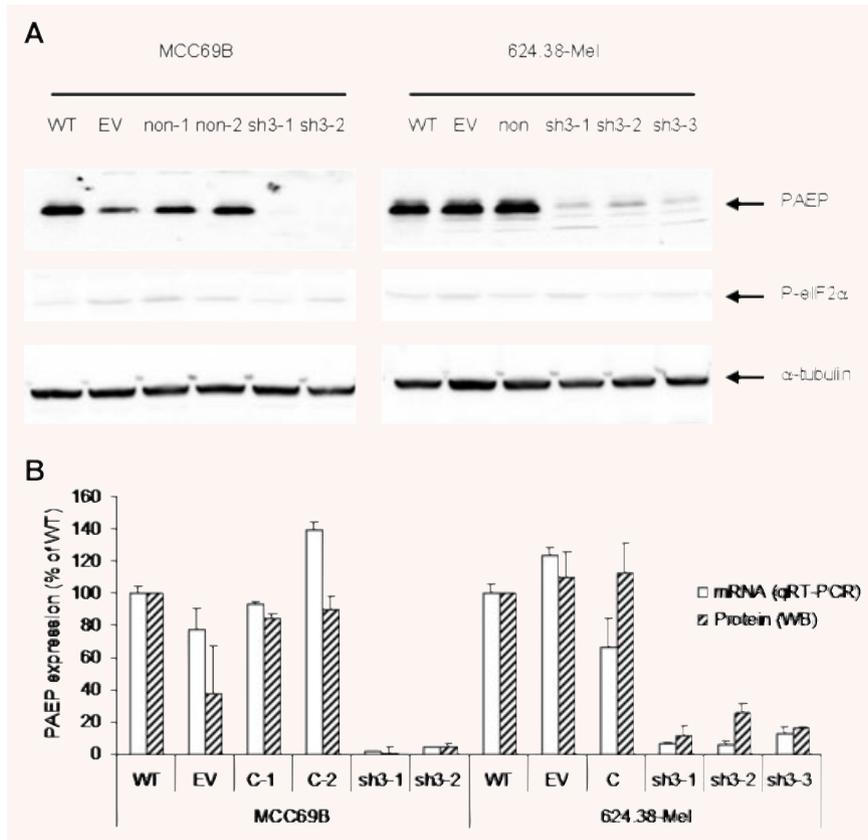


Fig. 4 Validation of melanoma shRNA stable cell lines. **(A)** Five MCC69B stable transfectants (1 empty vector, 2 shControl and 2 shPAEP3 clones) and five 624.38 stable transfectants (1 empty vector, 1 shControl and 3 shPAEP3 clones), together with their parental cells, were confirmed by Western blotting analysis. Protein samples were collected for PAEP and P-elf2- α examination from serum-free culture medium and whole cell lysate, respectively. α -tubulin was used as internal control. **(B)** PAEP expression in melanoma shRNA stable and parental cells measured by real-time qRT-PCR. The relative amounts of PAEP gene transcript were normalized by the reference gene GAPDH. The intensity of the bands above Figure 4A was analysed by Multi-Gauge V3.1 software. WT, wild-type; EV, empty vector; C, shControl; sh3, shPAEP3; -1, clone 1; -2, clone 2; -3, clone 3.

mRNA expression by 84% and inhibited tumour growth by 61%. The overall tumour sizes of shPAEP2 and shControl-1 groups were 3.1 ± 0.6 mm and 7.9 ± 0.3 mm, respectively.

Discussion

Melanoma is one of the most aggressive cancers in human beings, and despite immense efforts to understand its relative ease in its ability to metastasize, our knowledge remains limited as to the molecular and genetic changes that are involved in this process. One early hypothesis is the Clark model of melanoma progression, which describes a five-step sequence of events, beginning with a benign nevus transforming through a series of histological stages, eventually developing into a melanoma cell with subsequent metastatic capacity [16]. Recently, several high-throughput nucleic acid and tissue microarray platforms have been applied in order to identify molecular targets associated with both the molecular and phenotypic characteristics of melanoma from all stages of tumour progression. In doing so, we have been able to directly compare the gene expression patterns of representative melanoma samples derived from distinct pathophysiological states [32–34].

Previously, we have applied gene microarray analysis to compare the presumed genetic differences between the various states of progression of primary and metastatic melanoma samples derived from freshly procured tumour samples [23]. We found that there was a list of specific genes that were overexpressed and underexpressed throughout the various time-points of melanoma tumour progression, directly related to the overall Breslow's tumour thickness of the primary melanoma. When we compared a group of primary melanomas to metastatic lesions, we further identified a higher level of PAEP gene expression in the latter [24]. The high mRNA and protein expression of the PAEP gene was further validated in freshly procured melanoma tissues and daughter cell lines. We believe that this is the first report to identify a comparative overexpression of the PAEP gene in thick primary and metastatic melanoma samples.

PAEP is a secreted glycoprotein, which was first isolated from the human placenta, amniotic fluid, pregnancy deciduas and seminal plasma [1]. The protein has been suggested to contribute to the maternal immunotolerance in embryogenesis by suppressing the activity of immune effector cells [4–7] and contraception by inhibiting the interactions between sperm and the zona pellucida [8]. However, a differently glycosylated isoform of PAEP is recently found to stimulate sperm-zona pellucida binding, indicating specific glycosylation patterns of PAEP exert different roles on

regulating sperm function [35]. In accordance with its physiological functions, PAEP is reported to be frequently expressed in gynecologic malignancies [9–12]. However, the true function and role of PAEP gene expression in tumourigenesis and tumour progression remain unclear.

Recently, PAEP was observed to stimulate tumour cell migration induced by HDACI in human endometrial adenocarcinoma [13] and to promote neovascularization mediated by VEGF [14]. Contrary to these results, several studies have suggested that PAEP expression is associated with differentiated epithelia and its expression induces cell differentiation, thereby reducing the malignant characteristics of cancer cells [10, 35]. They further show PAEP reduced the tumour growth of MCF-7 breast cancer cells *in vivo* and suggested that PAEP acts as a tumour suppressor in breast cancer [36]. These conflicting results were partially explained by the differences between applying linear PAEP peptide and conformational PAEP protein or specific glycosylation patterns. Therefore, several methods should be applied to validate the results at different levels, with the silencing of intact wild-type PAEP expression a more reliable method to examine the role of PAEP. Additionally, it is also possible that PAEP may exhibit multiple physiological roles that are imposed by different signalling pathways associated with different tumour types or cell lines.

We therefore explored the overall cellular and genetic function of the PAEP gene, suggesting that it may play a significant role in the enhancement of cellular tumour progression with possible oncogenic tendencies. We utilized RNA interference (RNAi) to further delineate the gene function of PAEP, a tool well recognized for its ability to suppress gene expression and subsequently highlight the downstream effects of such loss of function [37, 38]. We optimized siRNA transfection in melanoma cells, significantly suppressing PAEP expression by as much as 80% at both the post-transcriptional and translational level.

Neoplastic cells may greatly differ from their normal, non-neoplastic counterparts at both the cellular and molecular level. Central among these differences are a loss of cellular contact-inhibition, the acquisition of an infinite life span and the ability to form tumours in animal hosts. Freedman and Shin found that there was a general correlation between the tumourigenic potential of transformed cells *in vivo* and their ability to grow in an anchorage-independent manner *in vitro* [39]. Growth in soft agar can be used as a surrogate *in vitro* assay to gauge the overall transformation potential and tumourigenicity. Thus, we examined the functional role of PAEP on melanoma colony formation in a soft agar assay, finding a significant decrease in melanoma cell colony formation after PAEP siRNA transfection. Furthermore, a colorimetric MTT assay revealed a significant decrease in total melanoma cell numbers in siRNA-treated samples by 27–46%. However, when examining the melanoma cell proliferation in an anchorage-dependent manner by MTS assay, we did not observe a significant difference with PAEP siRNA transfection in the cell lines examined. This may indicate that PAEP is more likely to affect melanoma cells in an anchorage-independent state.

Cellular motility and migration are other primary characteristics of tumour cells with metastatic potential. Uchida and associ-

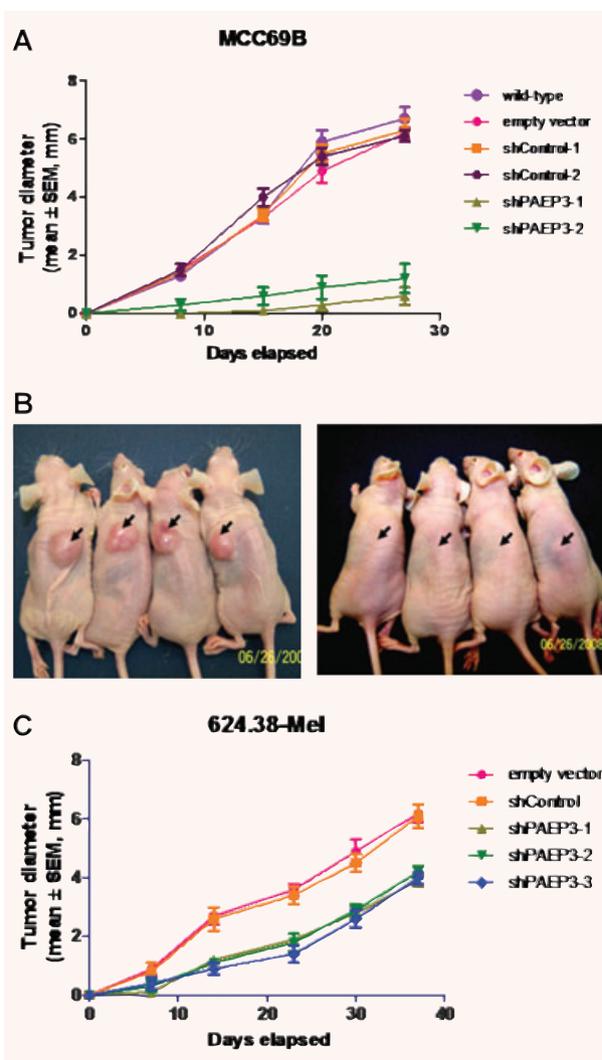


Fig. 5 Silencing of PAEP gene expression inhibits tumour growth in a xenograft model of human melanoma. (A) 2.0×10^6 MCC69B derived cells were subcutaneously injected into athymic mice (8 mice/group). Tumour size was measured weekly for 5 consecutive weeks and mean tumour diameter calculated by taking the square root of the product of orthogonal measurements. The tumour growth curve shows the mean tumour diameter relative to days elapsed. (B) Picture shows the xenografted melanomas in nude mice at the time of sacrifice. Four mice were chosen from each group for show. Left, MCC69B shControl-2; Right, MCC69B shPAEP3-1. ↓, the injection site. (C) The tumour growth curve of 624.38-Mel derived stable cells shows the mean tumour diameter relative to days elapsed.

ates convincingly describe the use of HDACI to stimulate cell migration in human endometrial adenocarcinoma cells, mediated by the up-regulation and overexpression of PAEP [13]. In our study, we directly knocked down PAEP gene expression in melanoma cells utilizing a siRNA approach. Subsequent scratch

assay and transwell assays revealed that both single and collective cell migration were significantly decreased after PAEP gene silencing. Furthermore, the invasion assay revealed that melanoma cells with high PAEP gene expression were more capable of penetrating the artificial Matrigel barrier. These observations lend support for a role of PAEP in cellular proliferation and migratory capacity in melanoma cells *in vitro*.

Establishing stable cells with PAEP expression silencing is necessary in order to further understand the potential role of PAEP in progressive tumour growth *in vivo*. To this end, we utilized a lentiviral vector that is capable of inducing a durable and stable silencing of PAEP gene expression [40]. Importantly, Western blot and real-time qRT-PCR analysis confirmed the expression of the PAEP gene in shPAEP stable transfectants, further showing a >75% and >90% knockdown of protein and mRNA, respectively. Lastly, a human melanoma xenograft model revealed a significant inhibition of melanoma growth initiation and tumour growth, with ~86% inhibition in MCC69B and ~33% in 624.38-Mel after silencing of PAEP gene expression.

Thus, our data strongly suggest that PAEP may represent a newly identified tumour-promoting gene, possibly an oncogene, in human melanoma and may serve as a surrogate target for gene silencing in melanoma patients. We further show that PAEP is capable of enhancing melanoma tumour progression by promoting cell proliferation and migration as shown through *in vitro* and *in vivo* analyses. Studies are currently underway to further understand the exact cellular and molecular mechanisms involved with PAEP gene expression as they relate to the mechanisms of tumourigenesis and tumour progression in human melanoma.

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