

RNAi-mediated downregulation of urokinase plasminogen activator and its receptor in human meningioma cells inhibits tumor invasion and growth

SHAKUNTALA KONDRAGANTI¹, CHRISTOPHER S. GONDI¹, IAN McCUTCHEON⁴,
DZUNG H. DINH², MEENA GUJRATI³, JASTI S. RAO^{1,2} and WILLIAM C. OLIVERO²

¹Program of Cancer Biology, Department of Biomedical and Therapeutic Sciences, and Departments of

²Neurosurgery, and ³Pathology, University of Illinois College of Medicine, Peoria, IL;

⁴University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

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Abstract. In recent years, RNA interference (RNAi) has emerged as an effective method to target specific genes for silencing. Several groups are actively exploring the use of small interfering RNA (siRNA) for therapeutic applications to treat cancer. Our previous studies have demonstrated the inhibition of various proteases, including serine proteases, cysteine proteases and matrix metalloproteases, via RNA interference (RNAi) in gliomas. Similar to gliomas, malignant meningiomas also exhibit elevated protease levels in comparison to normal brain and benign meningiomas. Here, we used siRNA to simultaneously target urokinase plasminogen activator (uPA) and its receptor, uPAR. A human CMV promoter-driven mammalian expression vector (pU2) was used to produce hairpin double-stranded RNA (hp RNA) to target uPA and uPAR. As determined by Western blotting and fibrin zymography, pU2 effectively inhibited uPAR protein levels and uPA enzymatic activity in meningioma cells (IOMM-Lee). *In vitro* studies (Matrigel invasion and spheroid migration)

revealed reduced meningioma cell invasion and migration. Intratumoral injections of the plasmid vector expressing siRNA for uPA and uPAR resulted in regression of pre-established, subcutaneous tumors in mice. In addition, *in vivo* studies of mice injected with pU2-transfected meningioma cells revealed inhibition of intracranial tumor formation. These findings suggest that siRNA can be used as a potent and specific therapeutic tool for the treatment of malignant meningiomas in humans.

Introduction

RNA interference (RNAi) is a genetic interference phenomenon in which double-stranded RNA (dsRNA) are processed to siRNA using Dicer, a cellular ribonuclease III, which generates duplexes of approximately 21 nucleotides with 3 overhangs (1,2). These siRNA are then incorporated into a silencing complex called RISC. When introduced into cells, dsRNA lead to the degradation of mRNA, which contain regions homologous to the triggering dsRNA. RNAi was initially seen in *C. elegans* (3,4) and *Drosophila* (5,6) as a protective mechanism against invasion of foreign genes. Elbashir *et al* (1) first reported that small interfering RNA (siRNA) (19-23 nucleotides) can effectively induce RNAi in mammalian cells. siRNA cause gene silencing at the post-transcriptional level in a sequence-specific manner without any apparent cell toxicity (7). Furthermore, compared to antisense oligonucleotides, very few molecules of siRNA are needed for gene silencing (8). For all of these reasons, RNAi is a novel, potent alternative to other gene therapies.

Meningiomas are primary brain tumors arising from the meninges (linings of brain) and they constitute approximately 20% of brain tumors. They range from benign, which are more common, to atypical and anaplastic (malignant) (9,10). Malignant meningiomas are highly infiltrative and invade brain, dura-mater and bone. In general, the tumors cannot be completely excised, even after radiotherapy. Currently, no effective chemotherapy exists. As a consequence, meningiomas present a therapeutic challenge.

Typical of many malignant tumor types, proteases play a major role in the ability of meningioma cells to invade

Correspondence to: Dr W.C. Olivero, Department of Neurosurgery, University of Illinois College of Medicine, P.O. Box 1649, Peoria, IL 61656, USA

E-mail: olib@uic.edu

Abbreviations: siRNA, small interfering RNA; RNAi, RNA interference; dsRNA, double stranded RNA; hpRNA, hairpin RNA; CMV, cytomegalovirus; uPA(R), urokinase plasminogen activator (receptor); MMP, matrix metalloprotease; pU2, plasmid expressing hpRNA for uPAR and uPA; pU, plasmid expressing hpRNA for uPA; pUR, plasmid expressing hpRNA for uPAR; EV, empty vector; SV, scrambled vector; DiI, 1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; DiO, 3,3'-diiododecyl-oxa-carbocyanine perchlorate; ECM, extracellular matrix; FRBA, fetal rat brain aggregate

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other tissues. Tumor cell invasion is a complex phenomenon involving interactions between tumor cells, normal stromal cells, and the extracellular matrix (ECM), as well as the subsequent degradation of the ECM and tumor cell migration (11). Invasive tumor cells are known to produce ECM-degrading enzymes, such as the plasminogen activators (PAs), which belong to the family of serine proteases. Plasminogen activators convert plasminogen into plasmin and further regulate a cascade of proteolytic events. The effect of plasmin appears to be either the direct degradation of ECM components (e.g. fibronectin, laminin and proteoglycans) or an indirect effect via the activation of latent forms of matrix metalloproteinases (MMPs), which hydrolyze plasmin-resistant collagenous elements. Our previous studies of human meningioma samples have established the increased expression of uPA, MMP-2 and MMP-9 (12).

Urokinase plasminogen activator (uPA) is a 55-kDa serine protease implicated in tumor cell invasion, angiogenesis and progression (13). uPA is secreted as an inactive precursor and can be activated into its active, single chain enzyme form by a variety of proteases, including plasmin, cathepsins B & L, and trypsin-like enzymes (14). Active uPA binds to its high affinity receptor, uPAR (CD-87), a 45- to 55-kDa cysteine-rich glycoprotein, which is covalently attached to the plasma membrane via glycosyl phosphatidylinositol (15). uPA, when bound to its specific cell surface receptor uPAR, efficiently converts plasminogen to the active serine protease plasmin (16), which then initiates the destruction of various ECM proteins. Through interactions between uPAR and integrins, as well as ECM components such as vitronectin, uPA aids in cell migration and adhesion (17,18). Thus, with or without involving the proteolytic activity of uPA, the uPA-uPAR system plays an important role in a variety of physiological and pathophysiological processes requiring cell movement, including wound healing, angiogenesis and tumor metastasis (13,19,20). Previous findings suggest the involvement of the uPA-uPAR system in chemotaxis (21,22) and in the activation of intracellular signaling pathways leading to enhanced cell proliferation, adhesion and migration (23-25). Thus, the uPA-uPAR system participates in the regulation of a wide range of cellular activities (26,27).

It is well documented that high levels of uPA and uPAR are associated with poor prognosis in many cancers, and that mice deficient in uPA have smaller, less hemorrhagic tumors (28). Considerable evidence of the elevated levels of uPA and uPAR in tumor tissue demonstrates their fundamental role in tumor invasion and metastasis and provides a rationale for novel therapeutic strategies. Several technical methods affecting tumor growth and metastasis, such as antisense oligodeoxynucleotides, adenoviral vectors, monoclonal antibodies, toxins, natural and synthetic inhibitors, linear and cyclic uPA-derived peptides etc. targeting the uPA-uPAR system in cancer patients at the gene and protein level, have been explored (29).

We have previously reported elevated levels of uPA in human meningioma tissue samples (12). We have also shown that antisense uPA stable clones (30) and adenovirus-mediated transduction (Ad-uPAR) of the uPAR antisense gene construct (31) resulted in less invasion and no tumor formation in nude mice. Similarly, intracranial injection of glioma cells with the Ad-uPAR-uPA antisense bicistronic construct inhibited

invasiveness and tumorigenicity (32). RNAi for MMP-9 and cathepsin B (33) and RNAi for cathepsin-B and uPAR (34) reduced glioma cell invasion and angiogenesis both *in vitro* and *in vivo*. We report here that RNAi for uPA and uPAR reduced meningioma cell invasion and caused tumor regression in nude mice. Hence, siRNA may have therapeutic importance in the treatment of human meningiomas.

Materials and methods

Construction of hpRNA expressing plasmid. A pcDNA 3 plasmid with a human cytomegalovirus (CMV) promoter was used to construct the hpRNA-expressing vector. The following sequences were used: for uPA, agcttGagagccctgctggcgcgccat atataatggcgcgccagcaggctctca from 346 to 367 bases, and for uPAR, tccTaccagcagtgagagcgattatataataatcgctctccactgctg tag from 77 to 98 bases. Inverted repeat sequences were synthesized for both uPA and uPAR (*HindIII* sites at the 5' and 3' ends).

Cell culture and transfection conditions. An established human meningioma cell line, IOMM-Lee (kindly provided by Dr Ian McCutcheon, U.T. M.D. Anderson Cancer Center, Houston, TX), was used in the current study. The cells were maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA) in a humidified atmosphere containing 5% CO₂ at 37°C. IOMM cells were transfected with EV, SV, pU, pUR or pU2 using the Lipofectamine reagent (Invitrogen, Grand Island, NY) as per the manufacturer's instructions. After transfection, cells were incubated in serum-containing medium for 48 h.

Immunoblot analysis. After transfection of IOMM-Lee cells with mock, EV, SV, pU, pUR or pU2 for 48 h, cells were collected and total cell lysates were prepared by incubating the cells in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA and 50 mM Tris, pH 7.4) for 1 h at 41°C. In total, 30 mg of protein from each sample was subjected to 12% SDS-Tris-glycine gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad Labs, CA). The membrane was blocked with 5% non-fat dry milk, 0.1% Tween-20 in PBS for 1 h. The primary antibodies employed were directed against uPAR (R&D Systems, Minneapolis, MN) and uPA (Biomed, Foster City, CA) followed by secondary antibodies (HRP-conjugated goat anti-mouse IgG+IgM, Biomed). The membranes were developed according to the manufacturer's protocol (Amersham, Arlington Heights, IL). For loading control, the membranes were stripped and probed with monoclonal antibodies for β-actin.

Zymography. IOMM-Lee cells were transfected with mock, EV, SV, pU, pUR and pU2. Conditioned media were collected and zymography performed as described previously (30,35). The samples were subjected to SDS-PAGE with 10% gels that contained fibrinogen and plasminogen. The gels were then washed twice with 2.5% Triton X-100 for 30 min each, incubated with 0.1 M glycine buffer (pH 7.5) at 37°C overnight, stained with amido black and then destained.

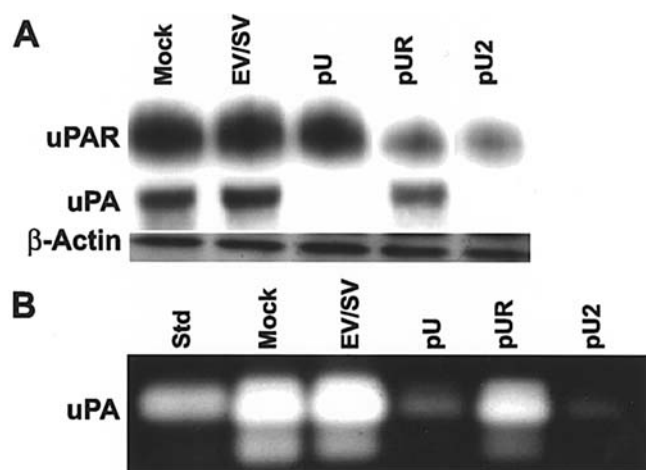


Figure 1. RNA interference decreased uPAR and uPA levels in IOMM-Lee cells. Total cell lysates and conditioned media collected from cells were transfected with mock, EV/SV, pUR, pU or pU2. Protein (10 μ g) was separated on 12% SDS-PAGE and immunoblotted with anti-uPAR antibody (A). Conditioned media (50 μ g) was separated on 12% SDS-PAGE and immunoblotted with anti-uPA antibody (A). β -actin antibody was simultaneously immunoprobed for loading control. Fibrin zymography was performed as described in Materials and methods with 1 μ g of conditioned media to detect uPA activity (B).

Matrigel invasion assay. *In vitro* invasion of meningioma cells was measured by the invasion of cells through Matrigel-coated (Collaborative Research Inc., Boston, MA) transwell inserts (Costar, Cambridge, MA) according to a previously described procedure (36). Briefly, 12-well transwell inserts with an 8-mm pore size were coated with a final concentration of 1 mg/ml of Matrigel in cold serum-free DMEM. Cells were trypsinized and 200 μ l of cell suspension (1×10^6 cells/ml) from each treatment were added to triplicate wells. After a 24-h incubation period, the cells that passed through the filter into the lower wells were quantitated by counting random fields and expressed as a percentage of the sum of the cells in the upper and lower wells (37).

Spheroid migration. A suspension of 5×10^4 cells in 100 μ l of Dulbecco's modified Eagle's medium was seeded on 96-well ultra low attachment culture plates (Costar) and continuously shaken at 40-60 r.p.m for 5 days until spheroids formed. Spheroids were then transfected with mock, EV, SV, pU, pUR or pU2 and cultured for 48 h. Seventy-two hours after transfection, spheroids were placed in each well of vitronectin-coated (50 mg/ml) 8-well chamber slides (Lab-Tek) and cultured with 200 μ l of serum-free medium. Spheroids were incubated at 37°C for 24 h, after which the spheroids were fixed and stained with Hema 3 and photographed. Migration of cells from spheroids to monolayers was measured using a microscope calibrated with a stage and ocular micrometer and used as an index of cell migration.

Three-dimensional spheroid invasion model. Multicellular spheroids were cultured in 96-well low attachment plates (Costar) prepared in DMEM. Briefly, 5×10^4 cells were suspended in 100 μ l of medium, seeded onto 96-well ultra low attachment plates and continuously shaken at 40-60 rpm for 5 days. The spheroids were then transfected and stained

with the fluorescent dye, Dil. Similarly, 18-day-old fetal rat brain cells were seeded and cultured for 21 days and stained with the fluorescent dye, DiO. The tumor spheroids and fetal rat brain aggregates were then co-cultured and, at various intervals, serial 1-mm optical sections were obtained with the aid of a confocal laser-scanning microscope. DiI and DiO fluorescence (Molecular Probes Inc., Eugene, OR) were detected by an argon laser at 488 nm with a band pass filter at 520-560 nm (DiI) and a helium/neon laser at 543 nm with a long pass filter at 590 nm (DiO). As described previously, the remaining volumes of rat brain aggregates and tumor spheroids were quantitated at 24, 48 and 72 h (36).

Subcutaneous tumors. IOMM-Lee cells (5×10^6) were trypsinized and resuspended in 100 μ l of serum-free DMEM and injected into the right flank of an athymic female nude mouse. When the tumors reached a size of 4-5 mm (usually in 7-10 days), mock-, EV-, SV-, pU-, pUR- or pU2-transfected cells were injected intratumorally at a dose of 60 μ g/mouse on alternate days for a total of five doses. The mice were observed for 4 weeks, after which the tumors were excised and photographed. Tumor volumes were quantified and graphically represented.

Intracerebral injection. To examine the *in vivo* effects of RNAi in meningiomas, we transfected IOMM-Lee cells with mock, EV/SV, pU, pUR or pU2. Cells (0.5×10^6) were trypsinized, resuspended in serum-free medium, and injected into the brains of athymic female nude mice. Thirty mice were used with five per treatment group. The mice were anesthetized with an i.p. injection consisting of 50 mg/kg ketamine and 25 mg/kg xylazine and injected intracerebrally with a 10- μ l aliquot (0.5×10^6) of the specified cell type with the aid of a stereotactic frame as described previously (36). After 4 weeks, the mice were sacrificed via intracardiac perfusion, first with PBS and then with formaldehyde. The brains were removed and embedded in paraffin as per standard protocol. Sections were prepared and stained with H&E. Serial sections of the paraffin blocks were taken and approximate tumor volume measured as a product of the tumor area and the depth of the tumor.

Results

pU2 transfection downregulates uPAR protein levels and decreases uPA activity in IOMM-Lee cells. Tumor cell invasion is a characteristic feature of most malignant tumors. uPA and uPAR are serine proteases which play important roles in ECM degradation and, as such, aid in tumor cell invasion. We targeted these genes using a bicistronic vector expressing siRNA for uPAR (77-98 bases of human uPAR mRNA) and uPA (346-367 bases of human uPA mRNA) under the control of a human cytomegalovirus (CMV) promoter. As determined by Western blotting, transfection of IOMM-Lee cells with pUR and pU2 inhibited uPAR protein levels as compared to mock- and empty/scrambled vector-transfected controls (Fig. 1A). Immunoblotting with conditioned medium from pU- and pU2-transfected IOMM-Lee cells revealed a decrease in uPA protein levels (Fig. 1A). β -actin levels determined that equal quantities of protein were loaded in each lane. Fibrin zymography results revealed a reduction in

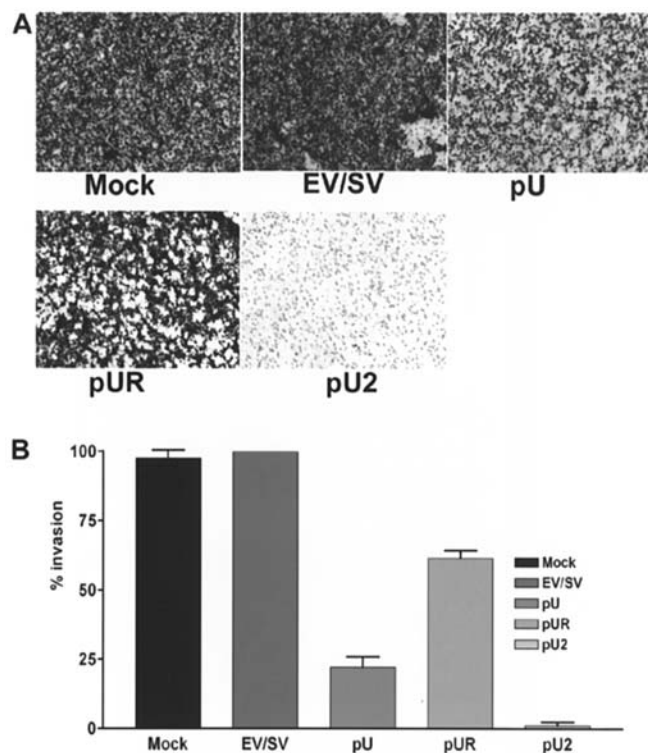


Figure 2. Matrigel invasion of IOMM-Lee cells transfected with mock, EV/SV, pU, pUR and pU2. 1×10^6 transfected cells were seeded on Matrigel-coated 12-well transwell inserts (8- μ m pores) and allowed to invade. After 24 h, cells that had passed through the filter into the lower wells were fixed and stained with Hema 3 and photographed (A). Percentage of invasion was quantitated as described in Materials and methods (B).

uPA activity in pU- and pU2-transfected cells as compared to mock-, EV/SV- and pUR-transfected cells (Fig. 1B). The decreases in uPAR expression and uPA activity are more pronounced in pU2-transfected cells, indicating that the bicistronic construct is more effective than either of the single constructs.

pU2 transfection decreases invasion of IOMM-Lee cells.

To study the effect of siRNA on the invasion capacity of meningiomas, 1×10^6 IOMM-Lee cells transfected with mock, EV/SV, pU, pUR or pU2 were seeded in the upper chambers of Matrigel-coated transwells. Twenty-four hours later, the cells which had migrated to the lower chambers were stained. Invasion of pU-, pUR- and pU2-transfected cells was low compared to mock- and EV/SV-transfected cells (Fig. 2A). Quantitative determination of invasion showed that only 0.3% of pU2-transfected cells invaded to the lower side of the membrane, 23% of pU-transfected, and 69% of pUR-transfected cells (Fig. 2B). As determined by the Matrigel invasion assay, inhibition of meningioma cell invasion was much higher in cells transfected with the bicistronic construct than either of the single constructs.

pU2 transfection of IOMM-Lee spheroids decreases migration.

Active cellular migration is essential for tumor invasion and metastasis. A number of factors, such as growth factors and cytokines, modulate migration. We performed a spheroid migration assay to study whether siRNA-mediated inhibition of uPA and uPAR can influence migration of meningioma

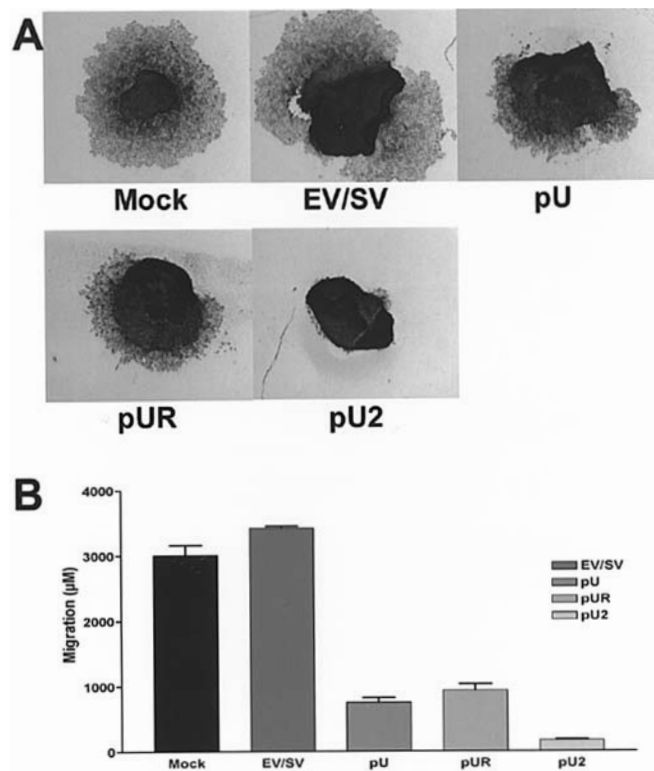


Figure 3. Spheroid migration by IOMM-Lee cells transfected with mock, EV/SV, pU, pUR and pU2. Spheroids of 100-200 microns were prepared by seeding 5×10^4 cells on 96-well low attachment plates by continuous shaking at 60 rpm. The spheroids were transfected with mock, EV/SV, pU, pUR or pU2. Seventy-two hours later, the spheroids were placed on vitronectin-coated 8-well chamber slides and allowed to migrate. After 48 h, the spheroids were then fixed and stained by Hema 3 (A). Spheroid migration was quantified and graphically represented (B).

cells. Multicellular meningioma spheroids grown in 96-well low attachment plates (100-200 μ m in size) were transfected with mock, EV/SV, pU, pUR, or pU2. Seventy-two hours later, spheroids were transferred to vitronectin-coated 8-well chamber slides and allowed to migrate for 48 h. The migrating capacity of the mock- and EV/SV-transfected spheroids was significantly higher than the spheroids transfected with pU, pUR and pU2 (Fig. 3A). Quantitative analysis (Fig 3B) showed that the control mock- and EV/SV-transfected spheroids migrated 2872 ± 30 μ m and 3200 ± 25 μ m. pUR-transfected spheroids migrated more than pU-transfected spheroids (872 ± 20 μ m and 780 ± 35 μ m, respectively), and pU2-transfected spheroids migrated the least (20 ± 5 μ m).

pU2 transfection of IOMM-Lee spheroids decreases invasion.

To study the siRNA effect of uPA and uPAR on IOMM-Lee cell invasion in a three dimensional system, meningioma spheroids stained with fluorescent dye DiI and fetal rat brain aggregates stained with fluorescent dye DiO were co-cultured. Staining of the cells with these dyes allows better visualization and characterization of the invasive pattern than other *in vitro* invasion assays. The advantage of this model is that tumor cells grown in three-dimensional culture closely resemble those of *in vivo* tumors. Tumor spheroids transfected with mock, EV/SV and pU2 were confronted with rat fetal brain aggregates in a 96-well agar-coated plate and observed at 24-, 48- and 72-h intervals. Co-culture assays revealed that tumor

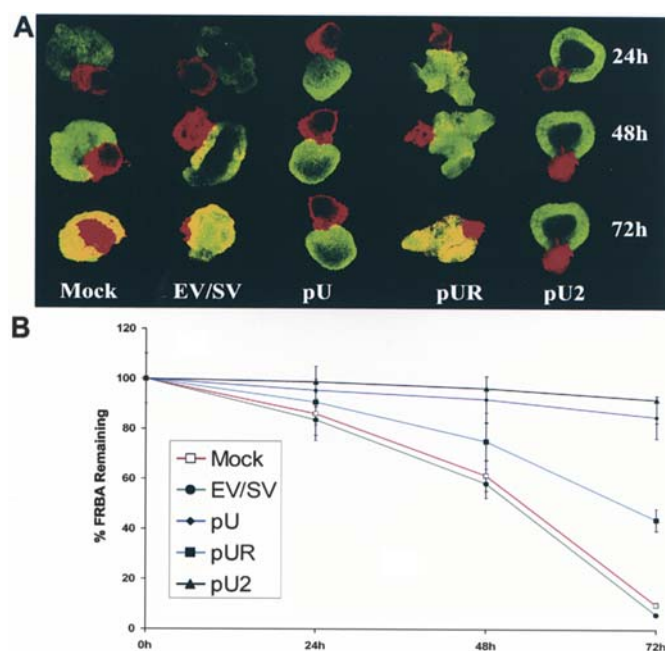


Figure 4. Spheroid invasion in IOMM-Lee cells transfected with mock, EV/SV, pU, pUR and pU2. Spheroids of 100-200 microns prepared as described in Materials and methods were transfected with mock, EV/SV, pU, pUR and pU2 and stained with DiI (red fluorescence). These spheroids were then co-cultured with rat brain aggregates stained with DiO (green fluorescence) obtained from 18-day-old fetal rat brains. Co-cultures were scanned with a laser-scanning microscope at 24, 48 and 72 h (A). Percentage of FRBA remaining after 24, 48 and 72 h was quantified and graphically represented (B).

spheroids treated with mock and EV/SV progressively invaded rat brain aggregates, causing almost complete invasion. In contrast, siRNA-transfected spheroids failed to invade rat brain aggregates (Fig. 4A). Quantitative determination of the percent of FRBA remaining was determined at 24, 48 and 72 h after confrontation (Fig. 4B).

pU2 treatment causes regression of subcutaneous tumors. To study whether pU2 can inhibit tumor growth, we examined

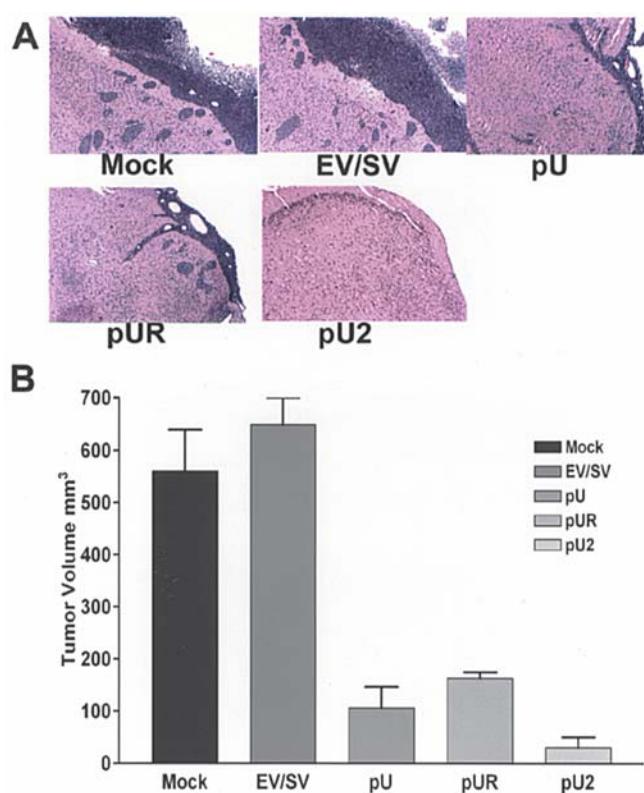


Figure 6. Suppression of intracranial tumor growth by RNAi. 0.5×10^6 IOMM-Lee parental cells or cells transfected with EV/SV, pU, pUR or pU2 were injected intracranially into nude mice. After 4 weeks, the mice were sacrificed, the brains removed and fixed in formaldehyde, paraffin-embedded and sectioned followed by H&E staining (A). The stained sections were viewed under a microscope and the tumor area determined in serial sections to approximately determine the intracranial tumor volume (B).

the ability of siRNA to inhibit pre-established tumors in nude mice. Subcutaneous tumors were developed by injecting 5×10^6 IOMM-Lee cells into the right flank of female nude mice. When the tumor reached 4-5 mm in size, the tumors were injected every second day with pU, pUR or pU2 for a

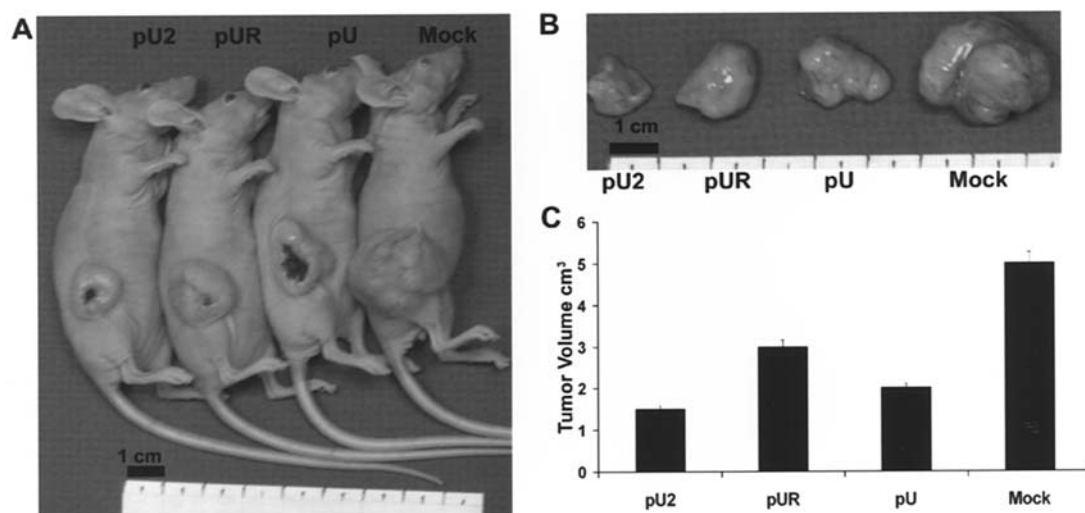


Figure 5. Regression of subcutaneous tumors by RNAi. 5×10^6 IOMM-Lee cells were injected subcutaneously into the right flanks of nude mice. When the tumors reached 4-5 mm in size, pU, pUR or pU2 was injected intratumorally on alternate days (A). A total of five injections were given. Tumors were excised after 4 weeks and photographed (B) and tumor volume was determined (C).

total of five doses (150 μ g of plasmid per dose in 100 μ l of sterile PBS). Mice were observed for 4 weeks, after which the tumors were excised. Fig. 5A and B shows regression of subcutaneous tumors injected with siRNA as compared to mock- and EV/SV-treated tumors. These results also demonstrate that inhibition of established tumor growth was much higher in mice treated with the bicistronic construct as compared to either of the single constructs. Tumor volume quantification (Fig. 5C) indicated a regression of tumor size in pU2-injected tumors.

Intracranial injections of pU2-transfected IOMM-Lee cells suppress tumor growth in mice. To confirm *in vitro* studies, we used an intracranial tumor model to assess the potential effects of siRNA-mediated suppression of invasiveness in nude mice. Half a million IOMM-Lee cells transfected with pU, pUR, pU2, and mock and EV/SV were injected intracranially into female athymic nude mice. Mice were sacrificed after 4 weeks. The brain sections of mice analyzed using H&E showed large spread tumor growth in mice injected with mock and EV/SV. In contrast, mice injected with siRNA-transfected cells showed minimal or no tumor growth, thus proving the effectiveness of RNAi in inhibiting meningioma invasion (Fig. 6A). Semi-quantification of serial tumor sections was performed and shows the regression of intra-cranial tumors in pU2-treated mice (Fig. 6B).

Discussion

RNAi has opened doors not only as a tool for gene therapy, but also for understanding the basic mechanisms underlying cellular function. RNAi is currently being exploited for its therapeutic potential in cancer, genetic diseases and viral diseases (38,39). Studies have demonstrated that dsRNA shorter than 30 nucleotides can be used for triggering RNAi without activating the mammalian interferon system (40). However, difficulties involving the efficient delivery of RNAi into mammalian cells (41) and the off-target effects (42) persist. Nonetheless, due to the great success of synthetic siRNA in mammalian cell cultures and its function *in vivo* (43), several laboratories are currently utilizing RNAi to target cancer cells and tumor growth (44,45).

Although meningiomas are some of the most common CNS tumors, they remain understudied in comparison to gliomas. Most meningiomas are resectable with the exception of meningiomas, which are malignant (3-5%) and atypical (9,10). For these recurring tumors, radiotherapy is not completely effective and no effective chemo-, hormonal or immunological therapies exist. For these reasons, we targeted these tumors with RNAi in this study. As the results demonstrate, we were able to effectively downregulate uPA and uPAR, and thus inhibit tumor growth, invasion and migration.

The characteristic features of any malignant tumor are tumor cell invasion and migration. Cell invasion is due to degradation of the ECM by proteases. These proteases are involved in tumor growth and invasion at both primary and metastatic sites (46), particularly at the invading foci (47). Our previous studies have demonstrated the roles of uPA and uPAR in malignant tumors whose interaction is crucial to invasion. Studies have also shown that using SNB19

antisense clones for uPA (30), uPAR (35,36), Ad-uPAR (31), and Ad-uPA-uPAR (32) as well as RNAi for cathepsin B and uPAR (34) decreased tumor growth and invasion in gliomas.

In this study, as assessed by Western blotting and fibrin zymography, transient transfection of IOMM-Lee cells with pU2 decreased endogenous uPAR levels and uPA activity. In addition, the results of our migration studies showed little or no migration of cells from spheroids transfected with pU2. However, the migration observed in pU- and pUR-transfected spheroids suggests that both uPA and uPAR are required for tumor cells to migrate.

Previous studies indicate that uPA participates in signal transduction pathways involved in adhesion and motility, independent of its proteolytic activity (29). uPAR is a glycosyl phosphatidylinositol-linked protein involved in multiple protein-protein interactions (48). uPA mediates cell migration and adhesion through interactions between uPAR and integrins as well as vitronectin (17,18). uPA has been shown to promote physical association between uPAR and α v β 5 integrins, which is required for uPAR-directed cell migration in breast carcinoma cell lines. uPA has also been shown to promote cytoskeletal rearrangements as mediated by α v β 5 integrins (49). Planus *et al* (50) reported that uPA and uPAR are involved in cellular migration. Our findings confirm that downregulation of uPA and uPAR decreases cell migration, possibly by cytoskeletal alterations.

The acquisition of invasiveness by tumor cells is important for tumor progression. There are several reports indicating the involvement of uPA and uPAR in tumor invasion. Disruption of interactions between these two at the cell surface results in blockage of activation of plasminogen and urokinase, inhibiting the proteolytic cascade required for invasion (51). uPA antibodies and inhibitors have been shown to inhibit tumor cell invasion into the ECM, amniotic and chick chorio-allantoic membranes (52-55). uPA antibodies also blocked metastasis in Hep3 human carcinoma cells in chick embryos and inhibited local invasiveness of subcutaneous tumors in nude mice (56). Previous studies in mice lacking the uPA gene showed retarded tumor development in a uPA-deficient environment (57). Our previous studies showed that antisense stable clones of uPA and uPAR were less invasive in *in vitro* models (30,35,36), as were clones that stably expressed an amino terminal fragment of uPA (ATF-uPA) (58).

In the present study, transfection with pU2 inhibited the invasiveness of IOMM-Lee cells and spheroids in Matrigel invasion and co-culture assays. In particular, downregulation of uPA and uPAR by RNAi significantly inhibited invasion of meningioma cells into surrounding ECM in Matrigel-coated transwells and into fetal rat brain tissue. This lack of invasion might be due to the inability of cells to anchor to the ECM. Hence, our studies confirm the significance of uPA and uPAR in the invasive behavior of meningioma cells.

Several studies have demonstrated that uPA antagonist peptides inhibit primary tumor growth in syngeneic mice (55). Kook *et al* (59) reported that antisense oligonucleotides decreased tumor growth, invasion and metastasis by reducing uPAR levels. Also, the downregulation of uPAR expression using an antisense strategy produced a protracted period of dormancy in human epidermoid cancer cells (60). The use of selective inhibitors of uPA and small, synthetic, cyclic,

competitive uPA antagonists derived from the binding site of uPAR resulted in reduction of tumor burden (61-63). Adenovirus-mediated downregulation of uPA and uPAR has also been shown to inhibit intracranial tumor growth in gliomas (32).

Our present study demonstrates regression of pre-established subcutaneous tumors and suppression of intracranial tumor growth in nude mice by pU2 when compared to mock and EV/SV vectors. These findings correlate with results from *in vitro* invasion studies and with studies demonstrating complete suppression of pre-established intracranial tumors by siRNA bicistronic constructs for cathepsin B & MMP-9 (33) and cathepsin B & uPAR (34) in gliomas.

In conclusion, the simultaneous downregulation of uPA and uPAR using RNAi successfully reduced meningioma cell invasion and migration *in vitro* and suppressed intracranial growth *in vivo*. The high tumor regression ability of the bicistronic construct compared to either of the single constructs suggests that RNAi of the uPA-uPAR system is required for effective therapy. These results indicate great potential for the siRNA-mediated downregulation of uPA and uPAR in therapeutic applications for treatment of malignant meningiomas.

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