U1 Adaptors for the Therapeutic Knockdown of the Oncogene Pim-1 Kinase in Glioblastoma

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U1 small nuclear interference (U1i) has recently been described as a novel gene silencing mechanism. U1i employs short oligonucleotides, so-called U1 adaptors, for specific gene knockdown, expanding the field of current silencing strategies that are primarily based on RNA interference (RNAi) or antisense. Despite the potential of U1 adaptors as therapeutic agents, their *in vivo* application has not yet been studied. Here we explore U1i by analyzing U1 adaptor-mediated silencing of the oncogene Pim-1 in glioblastoma cells. We have generated Pim-1-specific U1 adaptors comprising DNA, locked nucleic acids (LNA), and 2'-O-Methyl RNA and demonstrate their ability to induce a Pim-1 knockdown, leading to antiproliferative and pro-apoptotic effects. For the therapeutic *in vivo* application of U1 adaptors, we establish their complexation with branched low molecular weight polyethylenimine (PEI). Upon injection of nanoscale PEI/adaptor complexes into subcutaneous glioblastoma xenografts in mice, we observed the knockdown of Pim-1 that resulted in the suppression of tumor growth. The absence of hepatotoxicity and immune stimulation also demonstrates the biocompatibility of PEI/adaptor complexes. We conclude that U1i represents an alternative to RNAi for the therapeutic silencing of pathologically upregulated genes and demonstrate the functional relevance of Pim-1 oncogene knockdown in glioblastoma. We furthermore introduce nanoscale PEI/adaptor complexes as efficient and safe for *in vivo* application, thus offering novel therapeutic approaches based on U1i-mediated gene knockdown.

Introduction

AMONG GENE SILENCING technologies, U1 small nuclear interference (U1i) represents a novel alternative to RNA interference (RNAi). In this naturally occurring mechanism, the U1 small nuclear RNA (snRNA) is incorporated into the so-called U1 small nuclear ribonucleoprotein (snRNP) complex and hybridizes to the target precursor messenger RNA (pre-mRNA). Subsequent inhibition of poly(A) tail addition and pre-mRNA maturation leads to pre-mRNA degradation in the nucleus (Gunderson et al., 1994; Beckley et al., 2001; Fortes et al., 2003). Recently, artificial U1 adaptors have been described as a novel class of small, noncoding bifunctional oligonucleotides that can be employed for the induction of U1i-mediated gene silencing (Goracznik et al., 2009). With their 5'-half, the 26- to 28-nt-long U1 adaptors are able to bind to the terminal exon of the pre-mRNA, while their 3'-domain is complementary to a sequence within the U1 snRNA. By selecting a target sequence of choice, they recruit the snRNP complex that comprises 10 proteins bound to the U1 snRNA to the target pre-mRNA (Fig. 1). This leads to rapid and

selective target pre-mRNA degradation and thus decreased expression of the corresponding gene (Gunderson et al., 1998). Despite the highly specific knockdown of a target gene by U1 adaptors, they may also exert off-target effects, and conflicting results exist as to whether U1 adaptors generally interfere with splicing by sequestering snRNPs from the normal splicing process and/or lead to the knockdown of nontarget genes (Goracznik et al., 2009; Vickers et al., 2011). The efficacy of specific gene silencing and the extent of off-target effects depend on target sequence, suggesting that, comparable to RNAi, the analysis of multiple U1 adaptors is required. Furthermore, variations in adaptor length and the introduction of locked nucleic acids (LNAs), 2'-OMe RNAs and DNAs into the oligonucleotide sequence was shown to enhance binding affinity, specificity and mismatch discrimination, and has led to the identification of optimal U1 adaptor structures (see Grünweller and Hartmann, 2009 for review).

To the best of our knowledge, this is the first study to explore the therapeutic application of U1 adaptors *in vivo*. The major issue for the use of a nucleic acid in a therapeutic situation is its delivery *in vivo* (Aigner, 2008). We recently

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FIG. 1. Schematic representation of the mode of action of U1 adaptors (light gray) in the induction of U1 small nuclear RNA interference (U1i). *Lower panel:* Sequences of the adaptors employed in this study. The numbers of the adaptors indicate the position on the Pim-1 (Proviral integration site for Moloney murine leukemia virus 1) messenger RNA (mRNA) where the first base of the U1 adaptor binds. Chemistry of the bases: DNA, upper case; locked nucleic acids (LNA), upper case, bold; RNA, lower case, 2'-O-Me RNA, lower case, italic.

introduced poly(ethylene imine)s (PEI) for the complexation of small nucleic acids like small interfering RNAs (siRNAs) or micro RNAs (miRNAs) (Urban-Klein et al., 2005; Hobel et al., 2010; Ibrahim et al., 2011). The formation of nanoscale complexes compacts and protects the nucleic acid against degradation and allows its cellular uptake by endocytosis of the nanoplex and its subsequent intracellular release from the endosome based on the so-called proton-sponge effect. Certain linear or branched low molecular weight PEIs thus represent an efficient and nontoxic platform for the therapeutic delivery of small RNA molecules, and have been explored in various preclinical *in vivo* studies (Gunther et al., 2010).

Pim-1 (Proviral integration site for Moloney murine leukemia virus 1) is a constitutively active serine/threonine-kinase (Amaravadi and Thompson, 2005; Qian et al., 2005), whose target proteins are involved in apoptosis, cell cycle regulation, cellular signal transduction, and transcriptional regulation, and are overall linked to cell survival (see e.g., Aho et al., 2004; Zhang et al., 2007). Acting as a proto-oncogene, it is overexpressed in several tumor entities (e.g., B-cell lymphoma, prostate cancer, colorectal cancer, or pancreatic cancer) and is linked to poor prognosis (Brault et al., 2010). In hematopoietic malignancies and prostate cancer, Pim-1 is known to promote tumor onset and progression (Shah et al., 2008; Brault et al., 2010) by contributing to malignant transformation of cells during tumorigenesis (Nawijn et al., 2011). Pim-1 inhibition or knockdown in prostate carcinoma and colon carcinoma led to antitumor effects (Hu et al., 2009; Zhang et al., 2010). There is first evidence that in glioblastoma Pim-1 is overexpressed in tumor tissue and the inhibition of the kinase led to significant reduction in cell proliferation. Glioblastoma multiforme (World Health Organization classification grade 4) is the most aggressive form of astrocyte tumors. Due to the high mitotic activity of the tumor cells and

their infiltration of the surrounding tissue, the patients' median overall survival is approximately 1 year (Adamson et al., 2009). Radiotherapy or chemotherapy with temozolomide can hardly prolong survival, since tumor cells often show resistance against this aggressive treatment (Haar et al., 2012). Thus, new treatment strategies are needed to improve survival and life quality of the patients.

Materials and Methods

Design of Pim-1-specific adaptors

The Pim-1-specific mRNA-binding domains of the U1 adaptors were designed by folding the terminal 1,200 bases of the Pim-1 mRNA *in silico* using mfold to identify potentially unpaired regions. From the 3' exon 6, 13- to 14-nt-long sequences were derived, and the Pim-1 specificity of the sequences was verified by BLASTN. The mRNA-binding domains were combined with the constant U1 domain comprising LNA and 2'-O-Me RNAs described previously (Goraczniak et al., 2009), and the sequence of the complete bispecific adaptors were checked for self-complementarity. LNA modifications in the mRNA-binding domains were introduced at about every third position for non-self-complementary bases as shown in Fig. 1. The negative control adaptor Adaptor scr was generated by scrambling the sequence of the mRNA-binding domain of Adaptor 1361, and it was verified by BLASTN that Adaptor scr has no target in the human transcriptome. For the design of the additional negative control adaptor U1scr, the U1 domain was scrambled, while the Pim-1-specific domain of Adaptor 1361 remained unchanged. This Adaptor U1scr controlled for potential antisense effects via RNase H mechanisms. U1 adaptors were purchased from IBA GmbH. siRNAs targeting Pim-1 (siPim-1: 5'-GGA ACA ACA UUU ACA ACU CdTdT(sense) and

5'-GAG UUG UAA AUG UUG UUC CdTdT (antisense)) or luciferase mRNA (negative control siRNA, siCtrl: 5'-CUU ACG CUG AGU ACU UCG AdTdT (sense) and 5'-UCG AAG UAC UCA GCG UAA GdTdT (antisense) were purchased from Thermo Scientific.

Cell culture and transfection

Cell lines PC-3 (prostate carcinoma) and U87 (glioblastoma) were purchased from the American Type Cell Culture Collection and authenticated by the vendor. Cells were maintained in a humidified incubator under standard conditions (37°C, 5% CO₂) in Iscove's modified Dulbecco's media (PAA Laboratories) supplemented with 10% fetal calf serum (FCS).

For transfection, 2×10^2 cells were seeded in a 96-well plate or 5×10^4 cells in a 24-well plate and incubated under standard conditions unless stated otherwise. In 96-well plates, 1 pmol of adaptor was transfected per well, with INTERFERinTM siRNA transfection reagent (Peqlab) according to manufacturer's protocol, and incubated for the indicated periods of time. In 24-well-plates, 30 pmol adaptor or siRNA were transfected per well, unless stated otherwise, using Metafectene[®] (Biontex Laboratories) according to the manufacturer's protocol and incubated for the indicated time periods of time.

RNA preparation and detection by quantitative polymerase chain reaction

Cells were seeded and transfected in 24-well plates as described above and incubated for 72 hours. To prepare total cellular RNA, phenol/chloroform extraction was employed using 250 µL TRI-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. One microgram of RNA was transcribed to complementary DNA (cDNA) using the Revert-AidTM H Minus First Strand cDNA Synthesis Kit (Fermentas) as described previously (Ibrahim et al., 2011). cDNA was diluted 1:10 with nuclease-free water. A LightCycler[®] 2.0 (Roche) was used to perform quantitative polymerase chain reaction (qPCR) using the AbsoluteTM qPCR SYBR[®] Green Capillary Mix (Thermo Scientific). All procedures were conducted according to the manufacturers' protocols with 4 µL cDNA (diluted 1:10), 1 µL primers (5 µM) and 5 µL SYBR Green master mix. A 15-minute pre-incubation at 95°C was followed by amplification for 55 cycles: 10 seconds at 95°C, 10 seconds at 55°C, and 10 seconds at 72°C. After incubation at 65°C for 15 seconds, heating to 95°C was followed by rapidly cooling to 65°C to record the melting curve for PCR product analysis. Parallel to Pim-1-specific primers, Actin-specific primers were run for each sample to normalize for equal mRNA/cDNA amounts. Target levels were determined using the formula $2^{\Delta CP(Pim-1) / 2^{\Delta CP(Actin)}}$ with CP=cycle number at the crossing point (0.3).

Western blotting

Cells were seeded and transfected in 24-well plates as described above and incubated for 72 hours. Then medium was removed, cells were washed once with phosphate-buffered saline (PBS), and 200 µL lysis buffer [1 mM ethylenediamine-tetraacetic acid (EDTA), 1% NP40 in PBS, protease inhibitor cocktail (EDTA-free; Roche Molecular Biochemicals)] were

added per well. After incubating for 15 minutes on ice, the suspension was transferred to Eppendorf tubes and sonicated for 20 seconds. Following centrifugation (13,000 rpm, 4°C, 20 minutes), the supernatant was transferred to a new Eppendorf tube. The protein concentration was determined using the Bio-Rad DCTM Protein-Assay (Bio-Rad) according to manufacturer's protocol, and 4× loading buffer was added (0.25 mM Tris-HCl, pH 6.8, 20% glycerol, 10% beta-mercaptoethanol, 8% sodium dodecyl sulfate, 0.08% bromophenol blue) to yield a 1× concentration. Onto 10% polyacrylamide gels, 40 µg protein was loaded and separated by SDS polyacrylamide gel electrophoresis. After transferring the protein onto a 0.45 µm ImmobilonTM-P Transfer polyvinylidene difluoride membrane (Millipore), the blots were blocked with 5% (w/v) nonfat dry milk in Tris buffered saline with Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20). Blots were incubated with primary antibodies anti-Pim-1 (Epitomics) or anti-alpha-Tubulin (Sigma-Aldrich), diluted in 3% nonfat dry milk in TBST, at 4°C overnight. After washing with TBST, blots were incubated for 1 hour with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin (IgG) (Cell Signalling) or horseradish peroxidase-coupled goat anti-mouse IgG (Santa Cruz Biotechnology) in TBST with 3% (w/v) nonfat dry milk. After washing again with TBST, bound antibodies were visualized by enhanced chemiluminescence (ECL kit, Thermo Scientific). Scanned bands were quantitated using ImageJ (National Institutes of Health).

Proliferation and soft agar assay

To study anchorage-dependent proliferation, cells were seeded and transfected in 96-well plates as described above. The number of viable cells was determined using a colorimetric assay. At the time points indicated, the medium was aspirated and 50 µL of a 1:10 dilution of cell proliferation reagent water soluble tetrazolium 1 (WST-1) (Roche) in serum-free medium was added to the cells. After incubating for 1 hour at 37°C, the absorbance at 450 nm was measured using a Dynex MRX microplate reader (Pegasus Scientific Inc.).

For the assessment of anchorage-independent proliferation, soft agar assays were performed as described previously (Hobel et al., 2010). Briefly, cells were seeded in 6-well plates and transfected as described above. After 24 hours, cells were trypsinized and counted. Two hundred thousand cells in 0.35% agar (Carl Roth) were layered on top of 1 mL of a solidified 0.6% agar in a 6-well plate. The bottom layer contained 7.5% FCS and the top layer 8.5% FCS, respectively. Following a 1-week incubation, colonies >50 µm in diameter were microscopically counted by at least 2 blinded investigators.

Apoptosis assay

The rate of apoptosis was determined using the Caspase-Glo[®] 3/7 Assay (Promega). Five hundred HCT-116 cells were seeded in a 96-well plate and transfected as described above. Seventy-two hours later the medium was aspirated and 50 µL caspase substrate, diluted 1:5 in serum-free medium, was added to the cells. Following incubation for 1 hour at room temperature in the dark, luminescence was measured using a Fluostar Optima reader (BMG Labtec). A WST-1 assay was performed in parallel on the same plate to normalize for differences in cell densities.

Ethidium bromide exclusion assay

To study complexation efficiency and complex stability of PEI/adaptor complexes, ethidium bromide exclusion assays were performed. For the assessment of complexation efficiency, adaptors were complexed with PEI F25-LMW at different mass ratios. Briefly, 0.6 µg adaptor was diluted in 10 mM HEPES /150 mM NaCl, pH 7.4, and incubated for 10 minutes at room temperature (r.t.); 0–12 µg PEI F25-LMW were dissolved in the same buffer and incubated for 5 minutes, prior to being added to the adaptor solution. After 30 minutes of complexation at r.t., loading buffer (50% glycerol, 0.1% bromophenol blue) was added to yield a 1× concentration. Complexes were separated in a 1% agarose gel containing ethidium bromide and bands were visualized under ultraviolet light. The stability of PEI/adaptor complexes was studied by heparin displacement. Complexes comprising 1.5 µg PEI F25-LMW and 0.2 µg adaptor (mass ratio 7.5:1) were prepared as described above. Zero to Ten units of heparin (1 Howell unit being equivalent to 0.002 mg heparin) were added and incubated for 10 minutes at r.t. After addition of loading buffer, complexes were separated in a 1% agarose gel containing ethidium bromide. Bands were visualized under ultraviolet light, and their intensities were quantitated using ImageJ and plotted as dots per inch.

Atomic force microscopy

Atomic force microscopy imaging was performed using a Molecular Force Probe 3D instrument from Asylum Research in conjunction with an inverted optical microscope (Olympus IX 71, 40×). Silicon nitride V-shaped cantilevers with an average spring constant of 0.01 N/m and a pyramidal ultrasharp tip with a radius <20 nm (Veeco Instruments, model MSCT-AUHW) were employed. The spring constant of each cantilever was determined by the thermal noise method prior to each experiment (Sader et al., 1999). Mica (Plano GmbH) was chosen as a substrate because is atomically flat and requires mild preparation. Regular adhesive tape was used to “cleave,” or to separate adjacent sides, of a mica slide in order to produce a clean surface. Fifty microliters from the stock solution was deposited onto the surface of the mica slide and incubated for at least 30 minutes. Following incubation, the sample was rinsed 3–5 times with 1 mL buffer solution to detach any weakly bound complexes from the surface. Imaging was performed in regions of 2–5 µm, in contact mode at scanning rates of about 0.3 Hz. Imaging the surface with soft cantilevers allowed to apply normal forces within the range of 10–30 pN, thus reducing possible damage to the observed complexes by scratching or detachment due to shear forces.

Mouse tumor xenografts

Athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu}, 6–8 weeks of age) were obtained from Charles River and kept at 23°C in a humidified atmosphere and a 12-hour light/dark cycle, with standard rodent chow and water ad libitum. Experiments were performed according to the national regulations and approved by the local authorities (Landesdirektion Sachsen). Two million, five hundred thousand U87 cells in 150 µL PBS were injected subcutaneously into both flanks of the mice. When established tumors reached a

volume of 20–30 mm³, mice were randomized into specific treatment, negative control treatment, and nontreatment groups ($n=10$ tumors per group). For *in vivo* transfection, adaptors were complexed with PEI F25-LMW at the mass ratio 7.5:1. Briefly, 10 µg adaptor was dissolved in 75 µL 10 mM HEPES/150 mM NaCl, pH 7.4, and incubated for 10 minutes. Seventy five micrograms PEI F25-LMW was dissolved in the same buffer and, after incubation for 10 minutes, added to the adaptor solution. After briefly vortexing and incubating for 30 minutes at r.t., the complexes were aliquotted and stored frozen (Hobel et al., 2010). Prior to use, the complexes were thawed and allowed to incubate for 1 hour at r.t. For local treatment, 3 µg PEI F25-LMW-complexed adaptor was administered every 2–3 days by intratumoral injection. Tumor volumes were monitored every 2–3 days. Mice were sacrificed 1 day after the last treatment, and the tumors were removed. Pieces of the tumor tissue were fixed immediately in 4% paraformaldehyde and dehydrated in graded ethanols and xylene for paraffin embedding. The mouse blood was collected and allowed to clot prior to centrifugation at 13,000 rpm for 10 minutes to obtain the serum.

Immunohistochemistry

Paraffin-embedded sections were immunohistochemically stained for Pim-1 essentially as described previously (Hobel et al., 2010). Briefly, sections were deparaffinized with xylene and rehydrated with graded alcohols. Antigen retrieval was achieved by incubation in 1 mM EDTA, pH 8.0 at 90–95°C for 15 minutes. Endogenous peroxidases were inactivated by incubating the slides in 0.3% hydrogen peroxide in PBST at 4°C for 30 minutes. Blocking of the sections with 10% normal goat serum in PBST/2% bovine serum albumin for 1 hour at r.t. was followed by incubation with rabbit monoclonal anti-Pim-1 antibodies (Epitomics) in PBST, overnight at 4°C in a wet chamber. Slides were washed in PBST prior to incubation for 1 hour with a 1:1,000 solution of biotinylated horse anti-rabbit IgG (Vector Laboratories) in PBST. For visualization, sections were incubated with a streptavidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) for 30 minutes, followed by washing and incubation with 3,3'-diaminobenzidine. In the presence of immunoreactivity, a brown color was obtained on the section and the overall staining intensities were ranked observers from 0 (no staining) to 4 (strong staining) by blinded and expressed as “Pim-1 score.”

Liver enzyme activity assay

To assess the activity of liver enzymes, the diagnostic reagent kits from DiaSys (Holzheim) for alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (AP), and γ -glutamyltranspeptidase (γ GT) were used. The procedures were performed according to manufacturer's protocols. Absorbance at 340 nm (ASAT, ALAT) or 405 nm (AP, γ GT) was measured using a POLARstar Optima reader (BMG Labtec). As positive control, serum of a mouse treated with the liver-damaging bacterial endotoxin lipopolysaccharide (LPS) was employed.

Tumor necrosis factor alpha enzyme-linked immunosorbent assay

For the quantitation of *tumor necrosis factor alpha* (TNF α), blood was taken from mice 1 hour after intravenous injection

of 10 μ g PEI-complexed adaptor and analyzed by the Murine TNF α -ELISA Development Kit (PreProtech). The procedure was performed according to manufacturer's protocol using round-bottom Maxisorp 96-well microtiter plates (Nunc). The signal was developed with tetramethylbenzidine substrate (R&D Systems). After 30 minutes, the reaction was stopped with 2N H₂SO₄, and absorbance was measured in a microplate reader at 450 nm with the reference absorbance at 620 nm. Serum of mice treated with LPS was used as positive control.

Statistics

Statistical analyses were performed using Student's *t*-test or one-way analysis of variance/Tukey's multiple comparison post-test. Values are given as mean $\pm$ standard error of the mean.

Results

Based on the definition of unique Pim-1 binding sites and on the *in silico* prediction of U1 adaptor secondary structures that may mask U1 binding sites, 3 U1 adaptors to specifically inhibit the expression of Pim-1 were designed. As negative controls, structurally related U1 adaptors were employed, either with an unrelated scrambled sequence of the mRNA domain or with a functional mRNA domain but a scrambled U1 domain (Fig. 1). In U87 glioblastoma cells, all specific adaptors were able to inhibit Pim-1 mRNA expression, with Adaptors 1361 and 1495 showing the highest knockdown efficacy ($\sim$ 40%–55% residual Pim-1 levels; Fig. 2A, *left*). For direct comparison with RNAi, an optimal siRNA described previously (Thomas et al., 2012) was employed in parallel, and comparable knockdown efficacies of U1i (Adaptor 1361) or RNAi (siPim-1) versus the corresponding negative control (Adaptor scr or siCtrl, respectively) were observed (Fig. 2A, *left*). The Pim-1 knockdown was confirmed on protein level. Although comparable to siPim-1 in mRNA suppression, western blotting revealed that Adaptor 1361 suppressed Pim-1 protein levels somewhat less efficiently after 72 hours than siPim-1 (Fig. 2A, *right*). Similar U1i-mediated knockdown effects were observed in PC-3 cells and suggested a possible relationship between residual Pim-1 levels and adaptor dosage, especially when using the less efficacious Adaptor 1495, with a maximum 60%–75% knockdown versus Adaptor scr (Fig. 2B).

To control for potential Pim-1 knockdown via antisense effects mediated by RNase H, the negative control Adaptor U1scr, harboring the specific Pim-1 domain of the best-performing Adaptor 1361 and a scrambled U1 domain, was used. Upon transfection of U87 cells with Adaptor U1scr or Adaptor scr, no differences in Pim-1 mRNA levels were detected, whereas specific Adaptor 1361 led to a profound knockdown (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/nat).

Increased proliferation and evasion of apoptosis are the 2 key features of Pim-1 overexpression in tumor cells. Using the best adaptors from above, we next analyzed cellular effects of U1 adaptor-mediated Pim-1 knockdown. In line with the decreased Pim-1 levels observed, anchorage-dependent proliferation in PC-3 cells was substantially reduced upon transfection with Adaptors 1361 or 1495 (Fig. 3A). Contrarily, Adaptor scr did not mediate antiproliferative effects, thus excluding transfection artifacts. In U87 glioblastoma cells, the transfection with Adaptors 1361 or 1495 led to an even complete abolishment of anchorage-dependent proliferation. Again, the comparison with untreated cells also revealed the absence of nonspecific effects of Adaptor scr (Fig. 3B), which is in line with Pim-1 levels remaining unaltered (Fig. 2A, *left*). Furthermore, Adaptor U1scr was employed as additional negative control. Identical growth curves of untreated, Adaptor scr, and Adaptor U1scr transfected cells confirmed the absence of antisense effects (Supplementary Fig. 1B). Notably, the Pim-1-specific siRNA, although substantially reducing Pim-1 mRNA and protein level, only led to a moderate antiproliferative effect (data not shown). The antiproliferative effects of the Pim-1-specific Adaptors 1361 and 1495 were confirmed by impaired colony formation in soft agar assays that monitor anchorage-independent proliferation and thus resemble more closely the *in vivo* situation (Fig. 3C). Adaptor 1361 or 1495 transfected cells developed smaller and fewer colonies as compared to Adaptor scr transfected cells, leading to an overall reduction of colony formation by $\sim$ 50%. The induction of apoptosis was evaluated by measuring the activity of effector caspases 3 and 7. Upon delivery of Adaptors 1361 or 1495, a 3.5- to 4.5-fold increase in apoptosis in comparison with Adaptor scr was found (Fig. 3D). These findings indicate that Pim-1 is of functional relevance in glioblastoma and establishes U1i as potent mechanism for Pim-1 knockdown and identifies optimal Pim-1-specific U1 adaptors.

FIG. 2. (A) Comparison of adaptor-mediated versus small interfering (si)RNA-mediated knockdown of Pim-1 in U87 glioblastoma cells on mRNA (*left*) and protein levels (*right*). (B) Concentration-dependent reduction of Pim-1 mRNA, mediated by Pim-1 specific U1 adaptors in PC-3 prostate carcinoma cells. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; #, not significant.

FIG. 3. Cellular effects of adaptor-mediated Pim-1 knockdown in U87 cells. Anchorage-dependent proliferation in (A) PC-3 and (B) U87 cells as determined by water soluble tetrazolium 1 assay. (C) Soft agar assays for the assessment of anchorage-independent colony formation. (D) Induction of apoptosis as determined by caspase-3/-7 activity. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; #, not significant.

To further explore U1i with regard to possible therapeutic applications, we investigated the effects of U1 adaptor-mediated Pim-1 knockdown *in vivo* by treatment of subcutaneous glioblastoma xenografts in mice. As stated above, the efficient delivery of small nucleic acids to the target tissue represents a major bottleneck. As we have shown previously, polymeric nanoparticles based on the branched low-molecular-weight polyethylenimine PEI F25-LMW (Werth et al., 2006) are an efficient and nontoxic delivery platform for siRNA and miRNA, protecting the RNA from degradation and facilitating caveolae- or clathrin-dependent cellular internalization and endosomal escape. Additionally, PEI F25-LMW exerts favorable pharmacokinetic properties and high biocompatibility *in vivo* (Hobel et al., 2010; Ibrahim et al., 2011). First, to ensure the efficient incorporation of U1 adaptors into PEI F25-LMW-based complexes, the degree of adaptor complexation was analyzed at various PEI:adaptor mass ratios by increasing the PEI content in the complexation mixture. Complete complexation of the adaptor was seen already at a PEI:adaptor mass ratio of 1:1, as indicated by the absence of a free adaptor band in the sample subjected to gel electrophoresis (Supplementary Fig. S2A). For the assessment of complex stability, a heparin displacement assay was per-

formed. The addition of various amounts of heparin to the complexes revealed that a concentration of ≥ 1 U heparin was needed to displace the adaptors from the complexes, as subsequently analyzed by gel electrophoresis (Supplementary Fig. 2B). These results demonstrate that PEI F25-LMW-based adaptor complexation is equally efficient and PEI/adaptor complexes are equally stable when compared with siRNA (Hobel et al., 2011). We furthermore performed AFM to visualize PEI/adaptor complexes. AFM showed that the complexes are spherical and well shaped (Supplementary Fig. 2C), with ~ 40 – 60 nm in size as indicated by the signal heights. Again, this is comparable to previously analyzed PEI/siRNA complexes (Hobel et al., 2008) and led us to conclude that the complexation of adaptors with PEI F25-LMW may allow their *in vivo* application in a therapeutic setting, which was subsequently analyzed in a mouse tumor model.

To establish tumor xenografts, U87 cells were injected subcutaneously into athymic nude mice. Upon formation of tumors, mice were randomized into specific treatment (Adaptor 1495) and negative control groups (Adaptor scr, untreated). Adaptor 1495 was selected based on its most profound antiproliferative and apoptosis-inducing effects (see above). Three times a week for a period of 14 days, $3 \mu\text{g}$ PEI

FIG. 4. (A) Antitumor effects of polyethylenimine (PEI)/Adaptor 1495 complexes in a subcutaneous mouse tumor xenograft model versus Adaptor scr or untreated tumors. (B) PEI/Adaptor 1495-mediated Pim-1 knockdown in tumors, as determined by immunohistochemistry upon termination of the experiment. * $P < 0.05$; #, not significant.

F25-LMW-complexed Adaptor 1495 or Adaptor scr were injected intratumorally, and tumor growth was monitored regularly. Untreated tumors grew rapidly, increasing in volume by ~60-fold. Likewise, PEI/Adaptor scr-treated tumors showed profound tumor growth, slightly below the untreated group probably because of mechanical disruption of the tumor tissue by the injections, as observed previously (Ibrahim et al., 2011). Notably, however, upon treatment with PEI/Adaptor 1495 complexes, a marked tumor-inhibitory effect was observed, with a reduction in tumor volumes at day 13 after treatment start by ~40% and ~50% compared with negative control tumors and untreated tumors, respectively (Fig. 4A). For subsequent analysis, tumors were removed and analyzed for Pim-1 expression. The immunohistochemical staining of the tumor tissues for Pim-1 revealed a statistically significant ~35% inhibition of Pim-1 protein level in Adaptor 1495-treated tumors (Fig. 4B). This confirmed the Pim-1 specificity of the observed antitumor effect and highlights the biological relevance of Pim-1 in glioblastoma as well as the applicability of PEI/adaptor complexes for therapeutic intervention.

To demonstrate the *in vivo* safety of PEI/adaptor complexes with regard to the absence of liver toxicity and immune stimulation, we analyzed serum levels of established markers of liver damage—ASAT, ALAT, γ GT, and AP—and of the proinflammatory marker TNF α . No alterations in liver enzyme activities of ASAT, ALAT, γ GT, or AP upon repeated intratumoral injection of PEI/adaptor complexes were observed (Supplementary Fig. 3A, left and center). Likewise, in proinflammatory TNF α (Supplementary Fig. 3B), no significant changes were detected following intravenous injection of PEI/adaptor complexes (compare with LPS as positive control).

Discussion

The use of U1 adaptors for inducing U1i represents a novel gene-knockdown mechanism, thus extending the panel of sequence-specific gene silencing strategies and their potential therapeutic applications. While U1 adaptors have been shown to efficiently mediate the knockdown of a desired gene, their

off-target effects are discussed controversially (Goraczniak et al., 2009; Vickers et al., 2011). Dependent on the concentration and sequence of the adaptor, they may bind to sites of nontarget mRNAs with only incomplete complementarity or, by sequestering U1 snRNPs, interfere with splicing. This may eventually lead to the silencing of others than the target gene (Vickers et al., 2011). Some of these issues are reminiscent of the widely used RNAi, since both methods, U1i and RNAi, may interfere with essential, albeit different, cellular processes. Thus, despite a sequence-dependent mechanism of action, it is of great importance to closely control every U1 adaptor for nonspecific effects, and multiple adaptors need to be employed (see below). It should also be noted that while the siPim-1 used in this study decreased Pim-1 mRNA levels comparable to the transfection with Adaptor 1361, antiproliferative effects were less profound upon the RNAi-mediated Pim-1 knockdown. Beyond possible off-target effects, however, this discrepancy could be explained by different time kinetics, which would be relevant in an end-point determination of mRNA levels but not in a proliferation assay. The knockdown kinetics may also be influenced by the mode of delivery and intracellular processing of the given nucleic acid, which will in turn be determined by the transfection reagent. Taken together, the correlation between knockdown efficacies and biological effects may be more complex and not just reflect target gene specificity. It is also noteworthy that our negative control adaptors (Adaptor scr, Adaptor U1scr) never showed off-target effects other than those related to toxicity of the transfection reagent. Thus, the negative control Adaptor scr indicates that the observed Pim-1 adaptor effects are indeed sequence-specific and do not just rely on the presence of the U1 domain of the U1 snRNA. On the other hand, given that Adaptor U1scr comprises the specific Pim-1 domain and a scrambled U1 domain, a contribution of antisense effects mediated by RNAase H to the specific adaptor effects is unlikely as well.

In RNAi studies, it is instrumental to confirm the specificity of a given knockdown by the use of multiple specific siRNAs. The same can be expected to apply to U1i and therefore 3 specific adaptors were analyzed in this paper. This approach

also identifies the most efficacious sequences, bearing in mind that the efficacy will also depend on the accessibility of the respective target sequence. Indeed, different residual Pim-1 levels were observed. Whether additional selection rules for the identification of optimal adaptors apply—as seen before in the case of siRNAs where a very large body of knowledge has been accumulated over the last years—remains to be seen.

U1i is a silencing technique that, again comparable to RNAi, solely requires the introduction of oligonucleotides while all other components needed for the silencing machinery are provided by the cell. Chemical modifications of the U1 adaptors, for example, by introducing LNA modifications, can enhance their stability, affinity, and selectivity (Grunweller and Hartmann, 2009). This is the first study to employ nanoparticle-mediated delivery of U1 adaptors for a therapeutic application *in vivo*. The low-molecular-weight PEI F25-LMW (Werth et al., 2006) offers an excellent platform for their delivery, comparable to PEI/siRNA complexes (Hobel et al., 2010) despite the different structures of single stranded adaptors and double-stranded siRNAs. Since PEI F25-LMW is efficient for the induction of both U1i and RNAi, it will also be intriguing to explore the combined PEI-mediated siRNA and U1 adaptor delivery in 1 nanoscale complex. The simultaneous exploitation of 2 cellular mechanisms that take place in different cellular compartments may well exert additive or even synergistic effects on the knockdown of a given target gene.

The oncogenic kinase Pim-1 has been shown to be of high relevance in hematopoietic malignancies and prostate cancer, promoting tumor onset and progression (Shah et al., 2008; Brault et al., 2010). In other solid tumor entities, little is known about the role of Pim-1 in tumorigenesis. Recently, we reported the biological relevance of Pim-1 in colon carcinoma (Weirauch et al., 2013). In glioblastoma, the most aggressive form of brain tumors, there is first evidence of Pim-1 overexpression. In the present study, we show that U1 adaptor-mediated knockdown of Pim-1 leads to antiproliferation, induction of apoptosis and antitumor effects in glioblastoma cells. These findings provide the basis for further exploring the role and therapeutic potential of Pim-1 in glioblastoma. While several small molecule inhibitors for Pim-1 have been designed, their specificity and especially the discrimination between Pim-1 and Pim-3 has been a major issue (Brault et al., 2010). This limits the suitability of currently available Pim-1 inhibitors for target-orientated functional studies and therapies. Here, silencing based on U1i or RNAi are excellent tools for exploring the relevance of the Pim-1 oncogene and highly specific therapeutic strategies based on gene knockdown.

Acknowledgments

We are grateful to Anne-Katrin Krause and Nadine Beckmann for technical assistance and expert help with the experiments. This work was supported by grants from the German Cancer Aid (Deutsche Krebshilfe, grants 106992 and 109260 to A.G., R.K.H., and A.A.) and the Deutsche Forschungsgemeinschaft (DFG, AI 24/6-1 and AI 24/9-1 to A.A.).

Author Disclosure Statement

No competing financial interests exist.

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Received for publication November 23, 2012; accepted after revision April 15, 2013.