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Gene therapy with apoptosis-associated speck-like protein, a newly described schwannoma tumor suppressor, inhibits schwannoma growth in vivo

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Abstract

Background. We evaluated apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) as a schwannoma tumor suppressor and explored its utilization in a schwannoma gene therapy strategy that may be translated to clinical use.

Methods. ASC protein expression and mRNA level were assessed in human schwannoma by immunohistochemistry and quantitative PCR, respectively. Methylation- specific PCR was used to assess ASC promoter methylation. The effect of ASC overexpression in schwannoma cells was evaluated through ATP-based viability, lactate dehydrogenase release, and apoptosis staining. Western blotting and colorimetric assay were used to test the effect of ASC overexpression on endogenous pro-apoptotic pathways. Bioluminescence imaging, behavioral testing, and immunohistochemistry in human xenograft and murine allograft schwannoma models were used to examine the efficacy and toxicity of intratumoral injection of adeno-associated virus (AAV) vector encoding *ASC*.

Results. ASC expression was suppressed via promoter methylation in over 80% of the human schwannomas tested. ASC overexpression in schwannoma cells results in cell death and is associated with activation of endogenous caspase-9, caspase-3, and upregulation of BH3 interacting-domain death agonist. In a human xenograft schwannoma model, AAV1-mediated *ASC* delivery reduced tumor growth and resolved tumor-associated pain without detectable toxicity, and tumor control was associated with reduced Ki67 mitotic index and increased tumor-cell apoptosis. Efficacy of this schwannoma gene therapy strategy was confirmed in a murine schwannoma model.

Conclusion. We have identified ASC as a putative schwannoma tumor suppressor with high potential clinical utility for schwannoma gene therapy and generated a vector that treats schwannomas via a novel mechanism that does not overlap with current treatments.

Key Points

1. ASC expression is suppressed in human schwannoma via promoter hypermethylation.
2. ASC overexpression causes schwannoma death and cell-cycle arrest.
3. Intratumoral AAV1-P0-ASC injection reduces schwannoma growth in 2 in vivo models.

Importance of the Study

Schwannomas are tumors of the peripheral nervous system that cause debilitating neurologic deficits and can lead to death. Standard treatment includes debulking surgery that does not resolve the disease and can cause additional neurologic damage. Additional treatment strategies include γ-radiation and anticancer therapeutics in clinical trials that target the few pathways known to be disrupted in schwannomas. However, all are limited in scope and schwannoma treatment represents an unmet clinical need. Currently, there are no FDA-approved pharmacologic therapies for schwannomas. In this study, we report for the first time the discovery of a new potential suppressor of schwannoma development and the use of this tumor suppressor in a gene therapy vector that may be developed for clinical use. The relevance of this therapeutic option is that it is less invasive and safer than surgical resection and targets a novel pathway that does not interfere with standard and early-stage therapeutics.

Schwannomas are slow-growing tumors comprised of Schwann-lineage cells that develop along cranial and peripheral nerves.[1] In approximately 60% of individuals with schwannoma-associated disease, tumors initially appear in childhood and young adulthood, with new schwannomas continuing to develop in multiple locations throughout life.[2] Depending on location and size, these tumors can cause a variety of neurological symptoms including pain, deafness, tinnitus, dizziness, motor dysfunction (including paralysis), and even death due to brainstem compression.[3] In addition, while schwannomas initially present as benign and non-invasive tumors, they carry the potential to develop into malignant peripheral nerve sheath tumors.[4] Schwannomas may arise sporadically or as part of the debilitating genetic syndromes neurofibromatosis type 2 (NF2) and schwannomatosis. Current treatment of these tumors is largely limited to surgical resection, which carries significant risks and is not always possible because of risks to cranial nerves, peripheral nerve, spinal cord, and brainstem.[5] Specific risks of resecting vestibular schwannomas, the hallmark tumors of the NF2 syndrome, include cerebrospinal fluid leak, meningitis, bleeding, cerebral edema, and even death.[6] Clinical trials evaluating cancer chemotherapeutics, such as anti-angiogenic compounds and small molecule drugs, are ongoing, but efficacy of tested drugs has at best been limited and transient.[7] Moreover, bevacizumab, the drug that in clinical trials has demonstrated the most promising—albeit limited and transient efficacy for schwannoma treatment—is associated with substantial morbidity, including renal failure.[8] The paucity of therapeutic options for individuals with schwannomas reflects, at least partly, our limited knowledge of the biology and genetics that underlie schwannoma-associated diseases.

Schwannomas are appealing targets for gene therapy, as they grow slowly and can be readily localized using MRI. The advantage of gene therapy over surgical resection includes the fact that gene therapy strategies can utilize minimally invasive approaches, such as direct injection under MRI or ultrasound guidance, with minimal morbidity. We have previously described a gene therapy that safely targeted schwannomas using an adeno-associated virus (AAV) vector expressing a pro-apoptotic gene under the control of the Schwann-lineage cell-specific promoter P0.[9] Our published data clearly indicate that use of the P0 promoter, which is selectively expressed in Schwann-lineage cells, successfully prevents transgene expression in non-Schwann-lineage cells, including neurons, and tumor treatment with these vectors is not associated with any neural toxicity. While these data are promising, development of effective gene therapy approaches for schwannomas remain limited by our lack of knowledge of biologically relevant genes that act as key regulators of these tumors.

Apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)—known as ASC or PYCARD—is an adaptor protein that encodes a 23 kDa protein containing a pyrin domain (PYD) in the N-terminus and CARD in the C-terminus.[10] ASC activates

multiple apoptotic factors by mediating the assembly of large signaling complexes in apoptotic, inflammatory, and non-inflammatory pathways.[11] ASC promotes apoptosis by binding through its CARD site to caspase-1, caspase-8, and caspase-9.[11,12] These data suggest that ASC is an upstream factor regulating multiple apoptotic signals and thus an optimal transgene for use in tumor gene therapy. This hypothesis is further supported by the fact that *ASC*, which is also referred to as target of methylation-induced silencing (*TMS1*), was reported to be aberrantly methylated and silenced in multiple different cancers.[13,14] However, there are no data describing the expression of ASC in schwannomas or its relevance for the treatment of these tumors.

In this study, we show for the first time that ASC is specifically suppressed through methylation in both NF2-associated and sporadic schwannomas, suggesting a possible regulatory role for ASC in controlling the proliferation of these tumors. Based on this finding, we have characterized the effects of ASC overexpression in schwannoma cells and demonstrated for the first time that this protein induces apoptosis of schwannoma cells by activating caspase-3, caspase-9, and the pro-apoptotic BH3-interacting death domain agonist (BID), a pro-apoptotic member of the B-cell lymphoma-2 (Bcl-2) protein family.[15]These data clearly indicate the potential utility of *ASC* as a transgene for AAV-based schwannoma gene therapy. We have thus developed a novel AAV1-based gene therapy strategy that utilizes direct injection of our gene therapy vector into schwannomas to deliver the *ASC* transgene under control of the P0 promoter (AAV1-P0-ASC) to Schwann-lineage cells in a tissue-specific manner. The administration of this transcriptionally targeted vector leads to apoptotic death of schwannoma cells without apparent toxicity to other cells comprising the tumor-associated peripheral nerve, including neurons and resident Schwann cells.

Materials and Methods

Patients and Tissue Samples

Surgical vestibular schwannoma specimens were collected and processed per protocols approved by the Human Studies Committee of Massachusetts General Hospital and Massachusetts Eye and Ear.[16] The human NF2-associated VS tissues were obtained from Dr Anat Stemmer-Rachamimov, Pathology Core, MGH. Written informed consent was obtained from all subjects, and all procedures were conducted in accordance with the Helsinki Declaration of 1975. All human tissue utilized was de-identified and used with patient consent as per institutional policy.

Animals

All animal experimentation was approved by and conducted under the oversight of the MGH Institutional Animal Care and Use Committee. Animals, nu/nu and FVB/N mice, were kept on a 12:12 light-to-dark cycle with ad libitum access to food and water.

Cell Culture, Transfection, and Viability Assay

The HEI-193 human schwannoma cell line (from D.J. Lim, House Ear Institute) was established from a schwannoma in a patient with NF2, immortalized with human papillomavirus E6/E7 genes, and grown as described[17]; we validated the presence of the expected *NF2* gene point mutation (data not shown).[18] See Supplementary Material for further details.

Methylation-Specific PCR, Bisulfite Genomic Sequencing, and Real-Time PCR

Genomic DNA isolated from cultured cells was subjected to bisulfite conversion using the EZ-DNA Methylation Kit (Zymo Research) per manufacturer's directions. The modified DNA was used for methylation-specific (MS)-PCR analysis. See Supplementary Material for further details.

AAV Vectors

AAV1 serotype (AAV1) vectors were produced by transient co-transfection of 293T cells by calcium phosphate precipitation of vector plasmid (self-complementary [sc]) AAV1-rP0-hASC, scAAV-rP0-mASC, or scAAV1-rP0-null, adenoviral helper plasmid pAdΔF6, and a plasmid encoding the AAV rep gene and the AAV1 cap gene (pXR1), as previously described.[19] See Supplementary Material for further details.

Generation of Tumors and Vector Injection

Sciatic nerve schwannomas were generated by direct injection of HEI-193 human or 08031-8 mouse schwannoma cells into the left sciatic nerve of isoflurane-anesthetized athymic nude (nu/nu, 5- to 7-week-old males [National Cancer Institute]) or syngeneic FVB/N mice (5- to 7-week-old males [Charles River Laboratories]), as described.[20] Tumor growth was monitored by in vivo bioluminescence imaging, as described.[21] Details are provided in the Supplementary Material.

Behavioral Analysis

Behavioral testing utilized the von Frey method for pain/mechanical sensitivity, the Hargreaves plantar test for pain/thermal sensitivity, and rotarod for gross motor function, all according to published methods.[21,22] Nu/nu mice were used for the behavioral experiments. Details are provided in the Supplementary Material.

Histological and Immunohistochemical Analysis

Five weeks after intrasciatic injection with vector or control, animals were terminally anesthetized with isoflurane (3%) and sacrificed by decapitation. Sciatic nerves were removed and snap frozen for hematoxylin and eosin (H&E) and immunohistochemical staining, as described.[23] Details and antibodies used are provided in Supplementary Material.

Statistical Analyses

Data are presented as group averages ± standard error of the mean (SEM). Baseline behavioral values represent average of all measurements obtained before injection. In vitro cell culture data were analyzed with Microsoft Excel. Two-tailed *t*-test and repeated-measure ANOVA were utilized, as described.[24] $P < 0.05$ was accepted as significant. Pearson correlation coefficient analysis was conducted in the correlation experiments.

Results

ASC Expression Is Suppressed in Human Schwannomas

Immunohistochemical analysis of human tissues indicates that the high-intensity ASC protein expression observed in healthy peripheral nerve is decreased in the 6 NF2-associated schwannomas, with no two tumors resected from the same individual (Figure 1A, B). We next analyzed the level at which gene regulation may be occurring by performing real-time (RT) PCR to detect *ASC* mRNA. *ASC* mRNA expression was downregulated in schwannomas (Figure 1C) and cultured schwannoma-derived cells (Figure 1D) compared with healthy peripheral nerve and human Schwann cells. Further, MS-PCR was performed on the region covering the putative promoter and exon 1 of *ASC*, which is a typical cytosine-phosphate-guanine (CpG) island and therefore susceptible to epigenetic methylation and silencing. The same tumors that had decreased mRNA also showed *ASC* methylation (Figure 1E, F). Levels of *ASC* mRNA suppression correlated with increased methylation of the *ASC* promoter in both tumor and cultured cells (Figure 1G). Full methylation of the *ASC* CpG-rich promoter was demonstrated in 3 NF2-associated schwannoma samples (NF2 #1, 2, 3), and partial methylation was observed in the remaining 3 samples (NF2 #4, 5, 6; Figure 1E). This suggests the presence of multiple cell types (schwannoma and nontumor cells) with different levels of methylation in the latter 3 samples.

We found decreased *ASC* mRNA levels as well as complete methylation of the *ASC* promoter in the NF2 patient-derived HEI-193 cell line and in primary human schwannoma cells extracted from sporadic vestibular schwannomas (Figure 1F). This suggests that the methylation observed in tissues could be specific to the schwannoma-cell component of the tumors rather than to other cell types (for example, schwannomas can be rich in infiltrating macrophages).[25] Further validation of methylation density within the *ASC* promoter in HEI-193 cells was performed using bisulfite genome sequencing. The sequencing data were consistent with those obtained through MS-PCR and revealed frequent methylation of the *ASC* promoter in HEI-193 schwannoma cells, but not in primary human Schwann cells derived from healthy nerve (Supplementary Figure 1A, B). In addition, *ASC* promoter silencing was successfully reversed by treatment with the demethylating agent 5-aza-2-deoxycytidine (Supplementary Figure 1C).

To further determine whether ASC can be considered a key regulatory element in schwannoma growth, we analyzed *ASC* mRNA expression and promoter methylation in 2 healthy human peripheral nerves (sciatic and vestibular), 7 additional non-vestibular NF2-associated schwannomas, and 5 vestibular schwannomas. In all schwannoma samples with suppressed *ASC* mRNA, there was a correlated increase in promoter methylation (Supplementary Figure 2). While relative *ASC* mRNA expression was high in human peripheral nerve samples, 4 of 7 non-vestibular schwannoma samples and all 5 vestibular schwannoma samples showed decreased *ASC* mRNA and increased *ASC* promoter methylation (Supplementary Figure 2).

ASC Induces Apoptotic Death in Schwannoma Cells Grown In Vitro

We next investigated whether restoring ASC in schwannoma cells in vitro would affect tumor cell growth. We transfected human (HEI-193) and mouse 08031-9 or NF2S-1 (Supplementary Figure 3) schwannoma cell lines with a plasmid expressing *ASC* (both human and mouse genes were tested) under the regulation of the broadly active hybrid cytomegalovirus/chicken β-actin promoter (CBA) (Figure 2A). Twenty-four hours following delivery of the *ASC* gene, the *ASC*-transfected schwannoma cells showed a decrease in metabolic activity as measured by the ATP-based CellTiter-Glo assay and an increase in cell death as indicated by elevated lactate dehydrogenase (LDH) release (Figures 2B, C). Observed differences in cell viability between the 3 cell lines following ASC overexpression correlates with differences in transfection efficiency. In particular, HEI-193 had higher transfection efficiency (~51%), and greater ASC-mediated killing. The transfection efficiency of 08031-9 and NF2S-1 cells was substantially lower (~27% and 19%, respectively), and this was correlated with less cell death compared with HEI-193 following ASC overexpression (Supplementary Figure 4). Ectopic expression of ASC in normal human primary skin fibroblasts was not associated with increased cell death (Supplementary Figure 5). Flow cytometric analysis demonstrated significantly more ($P < 0.05$) double annexin V and propidium iodide staining (indicative of apoptotic cell death) of *ASC*-transfected HEI-193 cells compared with green fluorescent protein (*GFP*)-transfected or lipofectamine exposed cells (Figure 2E). ASC overexpression resulted in approximately 30% apoptotic/dead cells, compared with 5% and 4% for CBA-GFP transfected and lipofectamine exposed cells, respectively. This is consistent with the ATP-viability and LDH release results presented above. The effect of ASC overexpression on cell cycle arrest was examined using flow cytometry. Compared with *GFP*-transfected and lipofectamine control, *ASC*-transfected HEI-193 cells accumulated in G1 with a reduction in the percentage of cells in S and G2 phases. ASC overexpression increased the percentage of cells in the G1 phase from 52% for lipofectamine and 49% for GFP transfected cells to more than 71% (Figure 2F). These results suggest that ASC expression inhibited cellular proliferation of HEI-193 schwannoma cells via G1 arrest.

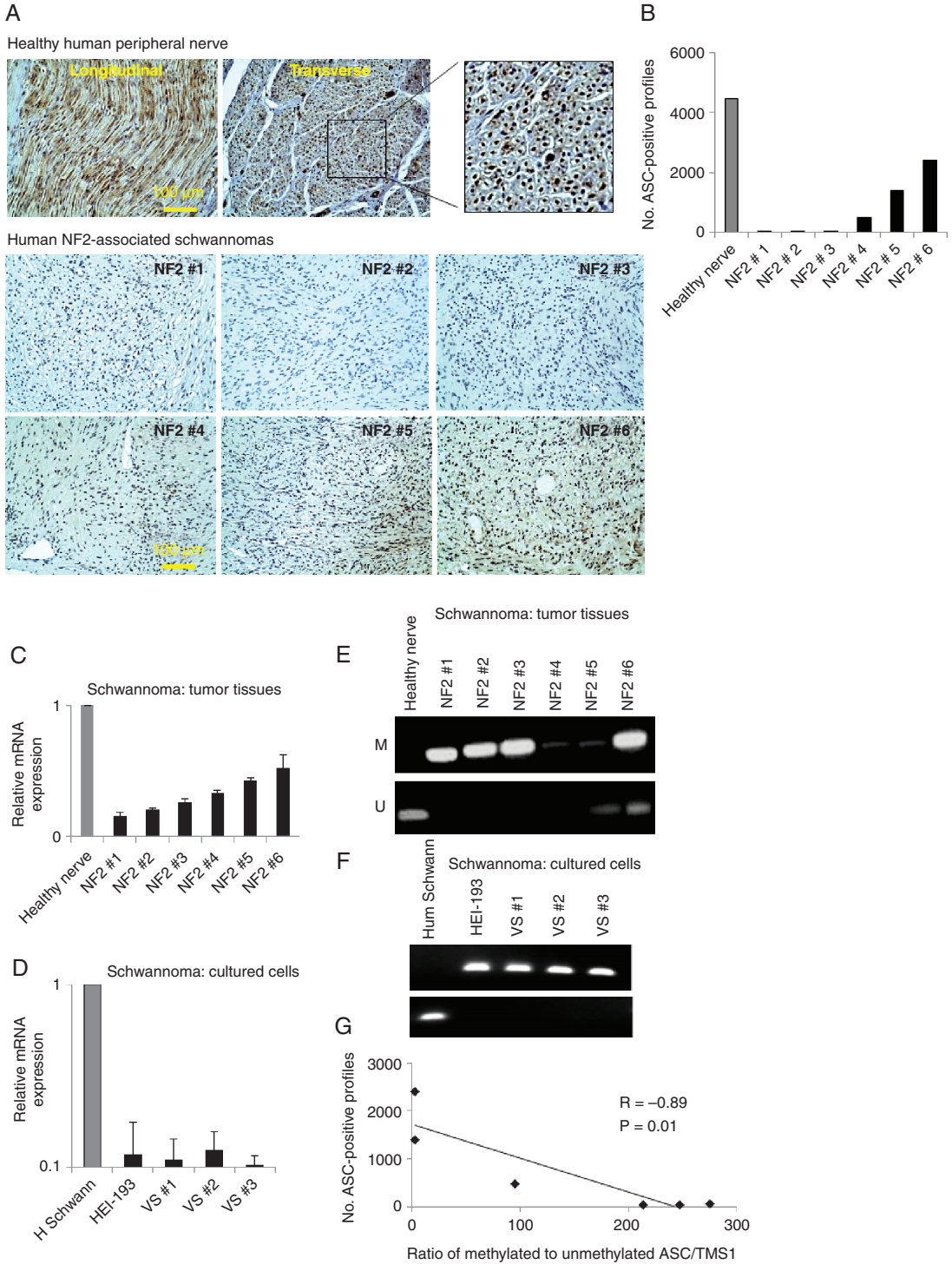

Fig. 1 ASC expression is decreased in schwannomas and this correlates with *ASC*-promoter methylation. (A) Representative immunostaining of human schwannoma specimens and healthy peripheral nerve. (B) Quantification of ASC immunostaining profiles per sample demonstrates greater numbers of ASC-positive cellular profiles in normal nerve compared with schwannoma. (C) Quantitative RT-PCR analysis of *ASC* expression in human schwannomas shows that *ASC* expression is downregulated in schwannomas with methylated *ASC*. (D) Quantitative RT-PCR analysis demonstrates that *ASC* expression in human Schwann cells, human HEI-193 schwannoma cell line, and human primary schwannoma cells is downregulated in cells with methylated *ASC*. (E) *ASC* methylation as determined by MS-PCR. (F) Methylation assay of *ASC* in cell lines (HEI-193 and 3 primary schwannoma lines). (G) Correlation chart showing that the downregulated schwannoma ASC protein expression levels are directly related to degree of methylation. (Quantitative PCR data are normalized to 18S gene and presented as fold increase, M: primers for methylated *ASC*, U; primers for unmethylated *ASC*.) R = Pearson correlation coefficient. Data in (C) and (D) are presented as means ± SEM.

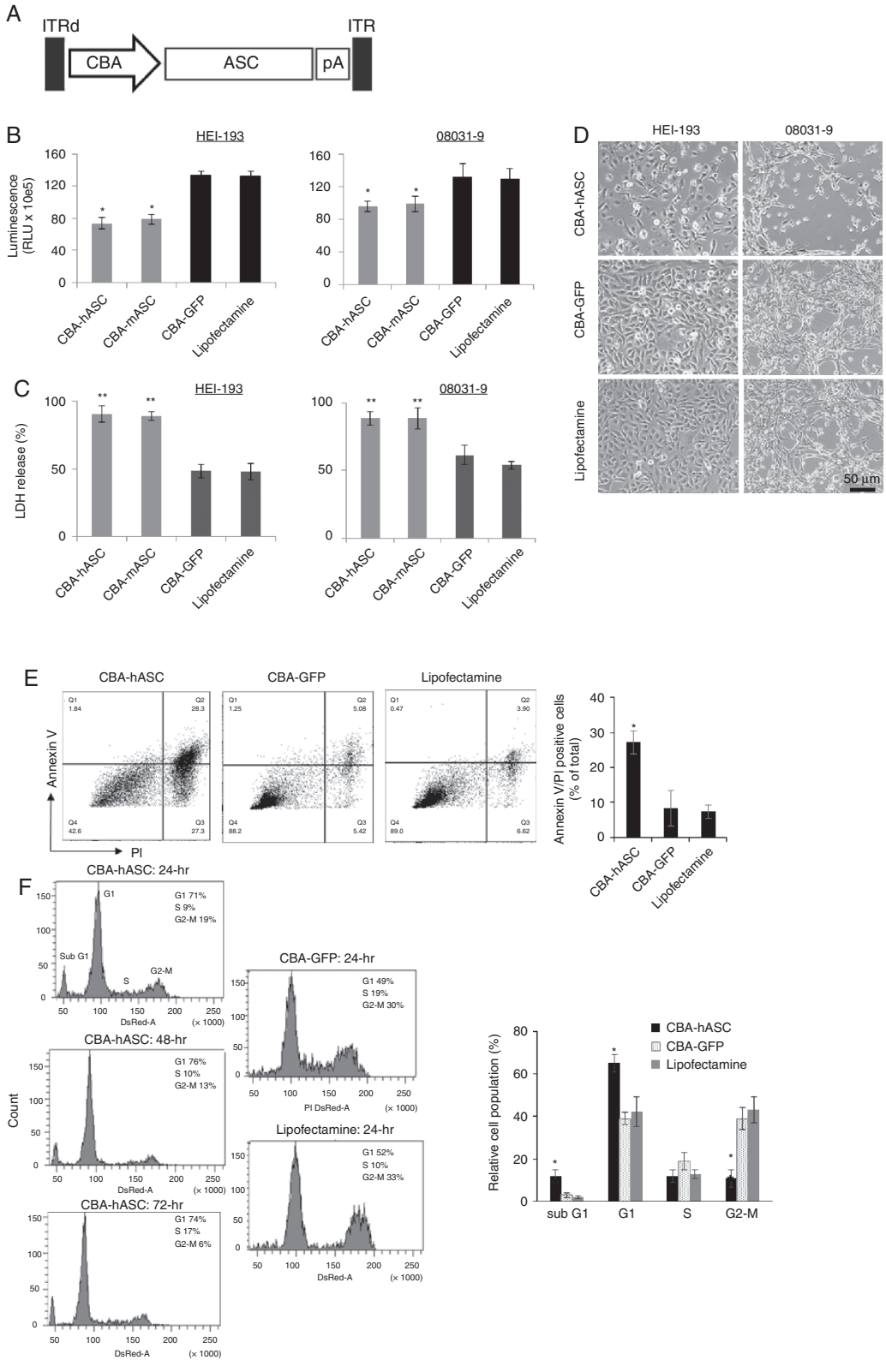

Fig. 2 In vitro ASC overexpression induces schwannoma cell death and cell-cycle arrest. (A) Schematic diagram of the self-complementary AAV vector encoding the human *ASC* (hASC) or murine *ASC* (mASC) gene under the control of the CBA promoter. (B) ATP-based viability of 2 schwannoma cell lines (human HEI-193 and mouse 08031-9) was measured 24 h following DNA transfection or lipofectamine exposure alone. (C) Lactate dehydrogenase (LDH) release cytotoxicity was measured at 24 h after transfection of human (HEI-193) and mouse (08031-9) schwannoma cell lines. (D) ASC

To determine whether ASC-mediated growth inhibition was the result of caspase-mediated apoptosis, we evaluated apoptosis in HEI-193 (human) and 08031-9 (mouse) schwannoma cells lines following overexpression of ASC. In these cells, ASC overexpression increased the number of apoptotic cells compared with vector transfected control (Figure 3A, B). Both a pan-caspase inhibitor (Z-VAD-FMK) and a caspase-3 inhibitor (Z-DEV-DFMK) blocked ASC-mediated apoptosis (Figure 3A, B). These data were consistent with western blot analyses performed on HEI-193 and 08031-9 cell lysates 24 hours following transfection with an AAV-based plasmid with *ASC* transgene under the control of a ubiquitous promoter ("pAAV-CBA-ASC"), control plasmid pAAV-CBA-GFP, or lipofectamine to determine expression and activation of a panel of apoptotic markers, including caspase-1, caspase-3, caspase-7, caspase-8, caspase-9, and BID. Western blot showed that in HEI-193 and 08031-9 cells ASC activated caspase-3 and caspase-9 and induced expression of BID (Figure 3C) but did not activate caspase-1 or caspase-8 (Supplementary Figure 6). These results correlate with specific caspase activity determined using a bioluminescent assay that quantifies caspase enzymatic activity. Caspase-3/-7 and caspase-9, but not caspase-1 and caspase-8, were found to be activated in HEI-193 schwannoma cells (Figure 3E and Supplementary Figure 6, respectively).

AAV1-Mediated *ASC* Delivery to Mouse Intrasciatic Schwannomas Decreases Tumor Burden and Associated Pain Behaviors

Based on our in vitro data, we next designed an AAV vector encoding human or mouse *ASC* (hASC, mASC) under the control of the rat P0 promoter for in vivo testing of therapeutic value (Figure 4A). Expression of the P0 promoter is restricted to Schwann and Schwann-lineage cells, the latter including HEI-193 cells in vivo (of note, P0 is minimally active in cultured HEI-193 cells; data not shown). We previously established the utility of P0 control in preventing toxicity, showing that P0 completely prevents transgene expression and associated tissue damage in neurons.[9] We generated 3 different AAV1 vectors, the experimental vectors containing hASC or mASC under control of the rat P0 promoter (AAV1-rP0-hASC, AAV1-rP0-mASC), and a control vector lacking a transgene sequence downstream of rP0 (AAV1-rP0-null).

Therapeutic efficacy of AAV1-rP0-hASC was first tested in a human xenograft model we developed.[20] In this model, schwannomas are generated in athymic nude (nu/nu) mice via intrasciatic implantation of a firefly luciferase (Fluc)-expressing variant of the NF2 patient-derived schwannoma cell line, HEI-193. Two independent experiments were conducted comparing intratumoral injection of AAV1-rP0-hASC with either AAV1-rP0-null or phosphate-buffered saline (PBS) vehicle as controls. To further validate efficacy of our therapeutic approach, we developed an allograft murine schwannoma model by implanting mouse 08031-9 schwannoma cells[26] in which we expressed firefly luciferase (Fluc) into the sciatic nerve of immunocompetent syngeneic FVB/N male mice. Growth curves were generated by implanting groups of mice with (1.E+03, 1.E+04, 3.E+04, 5.E+04, 1.E+05) 08031-9 schwannoma cells (Supplementary Figure 7). In this model, 2 independent experiments were performed, and we utilized mouse, rather than human, ASC in the viral vector (AAV1-rP0-mASC).

Tumor growth in the human xenograft and mouse allograft schwannoma models was assessed non-invasively using in vivo bioluminescent imaging.[20] Intratumoral injection of AAV1-rP0-hASC or AAV1-rP0-mASC led to a significant reduction in the growth of human schwannoma xenografts and mouse schwannoma allograft tumors, respectively, compared with null vector or PBS control (Figure 4B, C), as revealed by 2-way repeated-measures ANOVA. For the xenograft model, the magnitude of the effect was substantial; at the time of sacrifice (5 weeks following tumor injection for the xenograft model), there was a 4-fold higher bioluminescent signal in the AAV1-rP0-null and PBS control groups compared with the AAV1-rP0-hASC group. The reduction in HEI-193 tumor growth was confirmed by H&E staining of nerve sections, where a clear difference between experimental and control groups in tumor cell burden was evident (Figure 6A ix, x). AAV1-rP0-hASC mediated control of HEI-193 tumor growth began to wane approximately 3–4 weeks following vector injection (Figure 4B). This eventual tumor regrowth is likely due to loss of *ASC*-transgene copy number as tumor cells divide, rather than development of resistance to ASC effects, because transgenes delivered by AAV vectors are mainly maintained as extrachromosomal concatemers and do not efficiently integrate into host genome. This is further supported by the fact that intratumoral ASC protein diminishes over time following AAV1-rP0-hASC injection (Supplementary Figure 8).

By generating schwannomas that develop within the sciatic nerve, we can model the pain that is a common and major problem for individuals carrying these tumors. Indeed, we have previously reported that tumor expansion leads to mechanical sensitization, a pain behavior termed "allodynia" in humans and which can be used as an additional validation measure of tumor growth and consequently of therapeutic efficacy.[9] Thus, we used 2 behavioral tests to assess pain in mice treated with AAV1-rP0-hASC or AAV1-rP0-null. Thermal sensitivity was tested using the Hargreaves method, in which a movable infrared heat source is positioned underneath the hind paw ipsilateral to tumor and withdrawal latency is measured (Figure 5A).[27] Mechanical sensitivity (allodynia) was tested using von Frey filaments to establish withdrawal threshold of the hind paw ipsilateral to the tumor-implanted sciatic nerve

Fig. 2 *Continued*
overexpression via pAAV-CBA-ASC transfection in human HEI-193 or mouse 08031-9 schwannoma cells leads to altered morphology and cell detachment compared with GFP overexpression (pAAV-CBA-GFP transfection) or lipofectamine exposure. (E) Flow cytometric analysis demonstrated significantly greater double annexin V and propidium iodide (PI) staining of *ASC*-transfected HEI-193 cells. (F) PI cell cycle flow cytometry analysis of HEI-193 schwannoma cells following transfection-mediated overexpression of hASC or GFP, or lipofectamine exposure. Sub G1 represents apoptotic cells and is increased in CBA-hASC transfected cells. Graphical data are presented as means ± SEM ($n = 3$/group). $^{*}P < 0.05$, $^{**}P < 0.01$ (t-test).

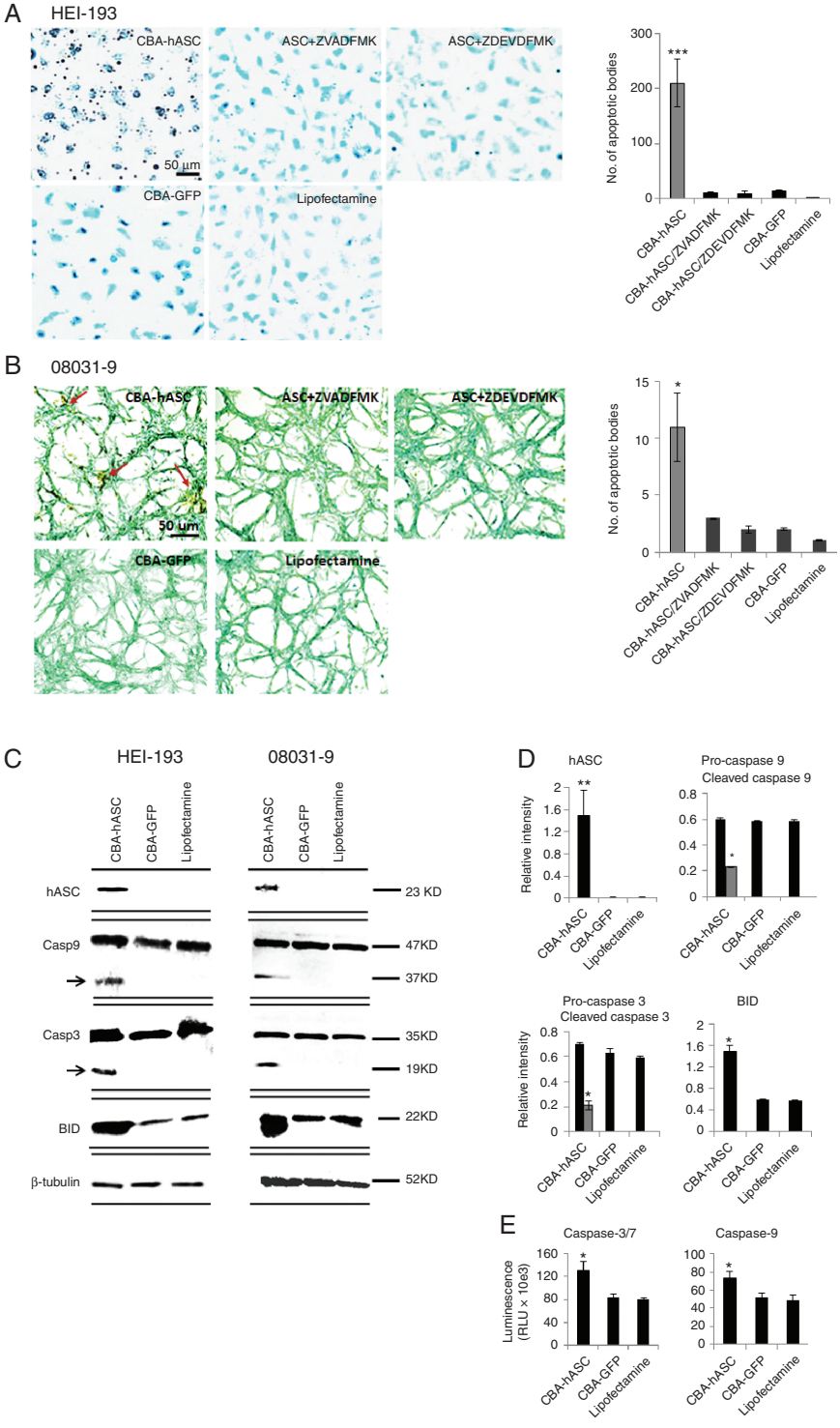

Fig. 3 ASC overexpression increases cell death in schwannoma cells in vitro. A, B) In vitro apoptotic body staining of HEI-193 (A) and 08031-9 (B) cells after transfection with pAAV-CBA-hASC plasmid shows obvious, brown-stained apoptotic bodies (red arrows). Apoptosis was abolished in presence of a pan-caspase inhibitor or caspase-3 inhibitor. (C) Western blotting of HEI-193 and 08031-9 cell lysates 24 h after transfection with pAAV-CBA-hASC plasmid compared with controls. (D) Quantification of HEI-193 western blot results demonstrated significant increases in cleaved (active) caspases-9 and -3, and in BID in ASC overexpressing HEI-193 cells. (E) Colorimetric assay demonstrated significantly increased caspase-3/-7 and caspase-9 activity in ASC overexpressing HEI-193 cells. Data are presented as means ± SEM (n = 3 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (t-test).

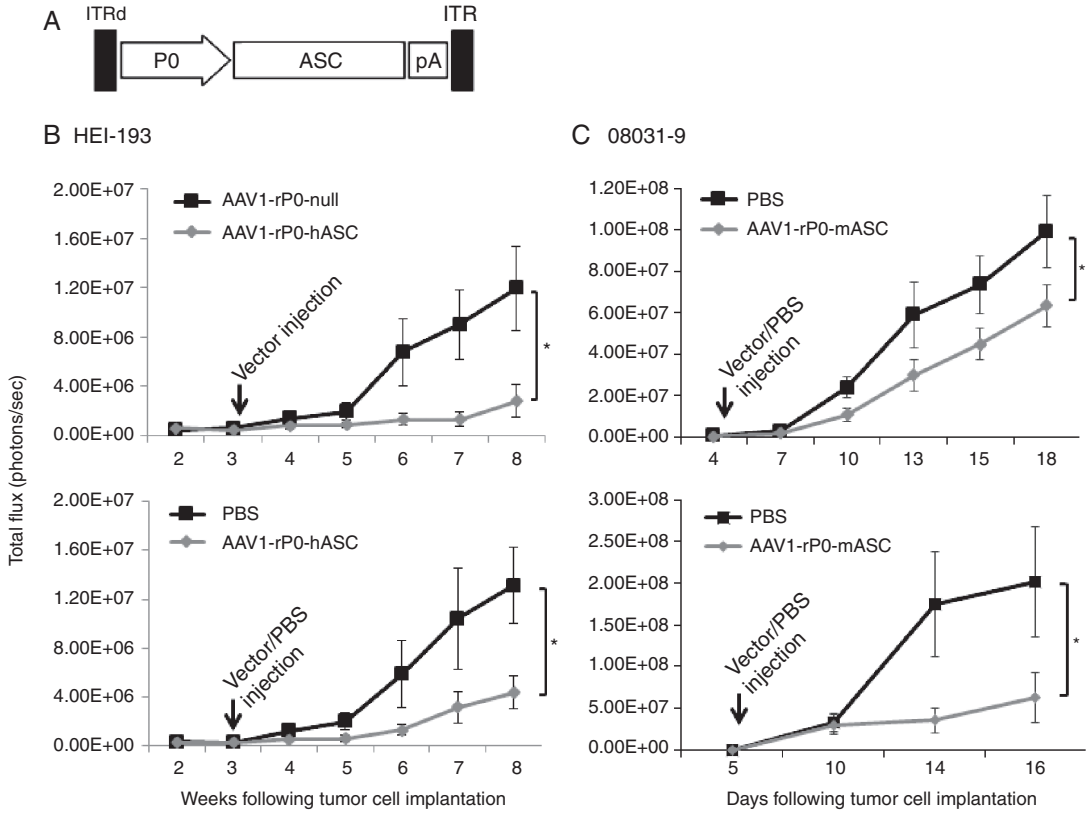

Fig. 4 AAV1-mediated expression of ASC in HEI-193 human or 08031-9 mouse schwannomas slows tumor growth in vivo. (A) Schematic diagram for self-complementary AAV1 vector encoding *ASC* gene under control of the Schwann-lineage cell-specific promoter, P0. (B) Intratumoral injection of the implanted xenografted mice 3 weeks post-implantation with either AAV1-rP0-hASC vs. AAV1-rP0-null vector (B, upper graph) or AAV1-rP0-hASC vs. PBS (B, lower graph). (C) Intratumoral injection of the implanted allograft mice 4 or 5 days post implantation with AAV1-rP0-mASC vs PBS control. A significant difference between AAV1-rP0-hASC and AAV1-rP0-null or PBS, and between AAV1-rP0-mASC vs PBS was observed by repeated-measures ANOVA (*$P < 0.05$). $N = 8$ mice per group.

(Figure 5B).[21] Gross motor performance, not a test of pain but a further behavioral measure of neurotoxicity, was assayed by the accelerating rotarod test (Figure 5C).[22]

In humans, schwannomas are commonly associated with severe, debilitating pain.[3] It is of obvious value for preclinical development to be able to behaviorally model schwannoma-associated pain and to evaluate the effect of any potential therapeutic not only on tumor burden but also on pain. In our xenograft model, schwannoma growth was associated with pain sensitization starting at 4 weeks post tumor cell implantation as demonstrated by a significant decrease in both the Hargreaves withdrawal latency (a measure of heat hyperalgesia) and the von Frey threshold (a measure of mechanical sensitization) in the hind paw ipsilateral to tumor (Figure 5A, B). This indicates that increasing tumor burden causes pain. For both behavioral pain tests ANOVA indicated a significant effect of intratumoral AAV1-rP0-hASC injection compared with AAV1-rP0-null (Hargreaves: $F(1,128) = 29.4$, $P < 0.0001$, von Frey: $F(1,128) = 33.4$, $P < 0.0001$), as well as a significant treatment by time interaction ($P < 0.005$ in both cases), the latter justifying post-hoc analysis. Gross motor function was not affected by tumor growth, nor did it change following treatment with AAV1-rP0-hASC (Figure 5C). Post-hoc analysis shows that the AAV1-rP0-hASC-mediated control of tumor growth (Figure 4B) was associated with a return of Hargreaves withdrawal latency and von Frey threshold values to baseline (ie, normalization of pain sensitivity) in these mice (Figure 5A, B). In contrast, post-hoc analysis for both Hargreaves and von Frey testing indicates that values for withdrawal latency (Hargreaves) and threshold (von Frey) in AAV-rP0-null tumor-injected mice remain significantly lower than baseline (ie, these mice continue to demonstrate pain sensitization) at all timepoints following vector injection. In summary, HEI-193 development within the sciatic nerve generates enhanced pain behaviors (heat hyperalgesia and mechanical sensitization) in the tumor-bearing hind limb, and AAV1-rP0-hASC treatment of these tumors is associated with normalization of these enhanced pain behaviors.

We then analyzed the cellular mechanisms through which AAV1-rP0-hASC decreased HEI-193 schwannoma growth in these mice. Histological comparison of tumors ($N = 3$ per vector) 5 weeks following injection with AAV1-rP0-hASC or AAV1-rP0-null indicated that tumors from mice expressing the *ASC* transgene had a decreased Ki67 index

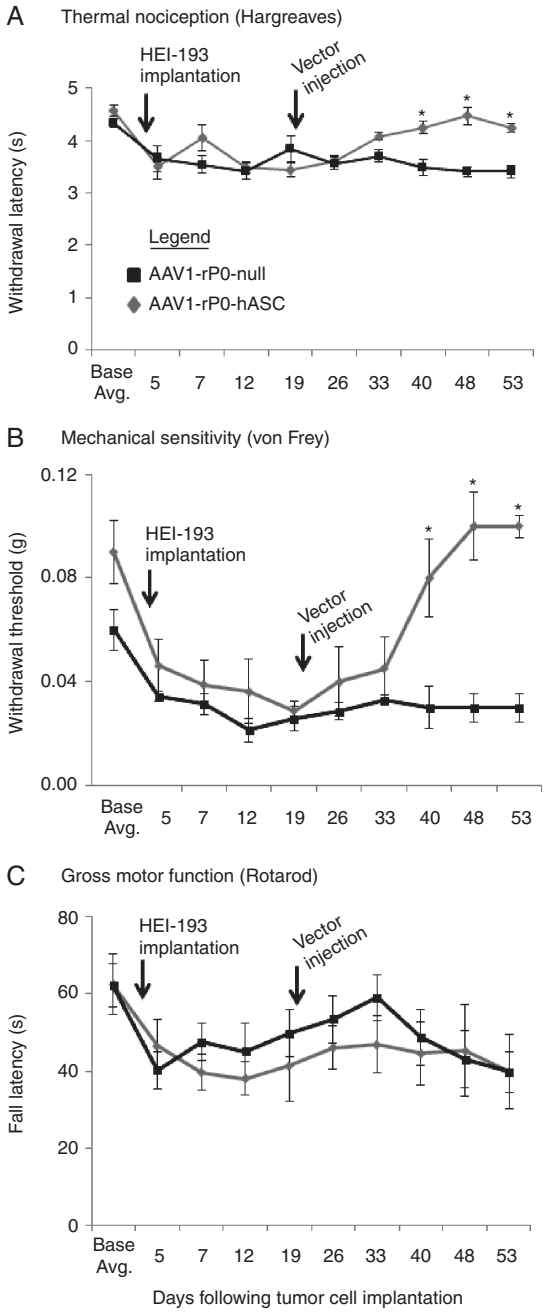

Fig. 5 AAV1-rP0-hASC treatment of HEI-193 schwannomas leads to resolution of tumor-associated pain behaviors and has no effect on gross motor function in vivo. Effects of AAV1-rP0-hASC or AAV1-rP0-null injection of implanted sciatic nerve HEI-193 schwannomas on pain behaviors, both thermal nociception (Hargreaves test, A) and mechanical sensitivity (von Frey method, B), and on gross motor function (rotarod, C) were evaluated at 2- to 7-day intervals for approximately 7 weeks following tumor cell implantation. Repeated-measures ANOVA with post-hoc testing was used to compare behaviors of the AAV1-rP0-hASC and AAV1-rP0-null injected mice with each other, as well as within each group comparing post virus injection at each time point with their respective baseline value. Results are represented as the mean ± SEM; $n = 8$ mice per group. $^*P < 0.05$.

(Figure 6A i, ii; quantification: 6B), suggesting decreased proliferation as well as an increased percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling positive cells, indicating increased apoptosis (Figure 6A iii, iv; quantification: 6B). In addition, the same tumors showed an increased expression of caspase-3 (Figure 6A v, vi) and caspase-9 (Figure 6A vii, viii). Western blot demonstrates abundant ASC protein in AAV1-rP0-hASC injected HEI-193 schwannoma-bearing sciatic nerve, but no detectable ASC in AAV1-rP0-null injected tumor-bearing nerve (Figure 6C).

Discussion

There is no truly efficacious drug therapy for schwannoma. The mainstay of treatment remains surgical resection, which is commonly associated with significant morbidity, and not always possible due to risks to cranial nerves, peripheral nerves, spinal cord, and brainstem[5]. This is the first study to propose a key role for *ASC* as a tumor suppressor gene in schwannomas. Based on this discovery, we have developed a safe and effective gene therapy vector for the treatment of these tumors. Such therapy will potentially permit minimally invasive schwannoma debulking in patients. In addition, even though we have not tested any combination treatments in this work, it is conceivable that AAV1-rP0-hASC may be combined with currently available treatments to achieve additive and potentially synergistic therapeutic effects. Importantly, the mechanism of action of AAV1-rP0-hASC is completely different from those of other schwannoma therapeutics currently being tested.

ASC is a bipartite protein that promotes apoptosis. It acts as an upstream factor of multiple apoptotic pathways[28] and activates multiple caspases through cleavage, such as caspase-8 and caspase-9.[12] ASC is also recognized as a key player in multiple tumor suppressor pathways that are considered hallmarks of cancer. Specifically, ASC activates the known tumor suppressor pro-apoptotic p53, upregulates BAX and BID, and suppresses the activity of the survival protein Bcl-2 in human breast cancer cell lines and leukemia.[29,30] Our data show for the first time that ASC also activates caspase-3, and at least in our schwannoma model this activation does not appear to be associated with toxicity. Neither the behavioral data nor the histopathological data indicate any neuronal damage; instead, an abrogation of tumor-induced pain is observed.

The crucial role of ASC as an upstream factor in multiple apoptotic pathways may explain why this gene is frequently found silenced through methylation in cancer tissues.[31,32] Previous work assessing gene methylation in 125 human schwannomas (spinal and vestibular) indicated the presence of 2 subgroups of tumors involving 395 differentially regulated genes.[33] This finding is consistent with an important regulatory role of gene methylation in schwannomas. We evaluated *ASC* methylation in a highly specific manner and showed that *ASC* is methylated in over 80% of the studied schwannomas, which were derived from patients affected with NF2-associated and sporadic schwannomas. ASC has never been proposed as a potential treatment for any benign neoplasm, and we are the first to develop ASC as part of any therapeutic product.

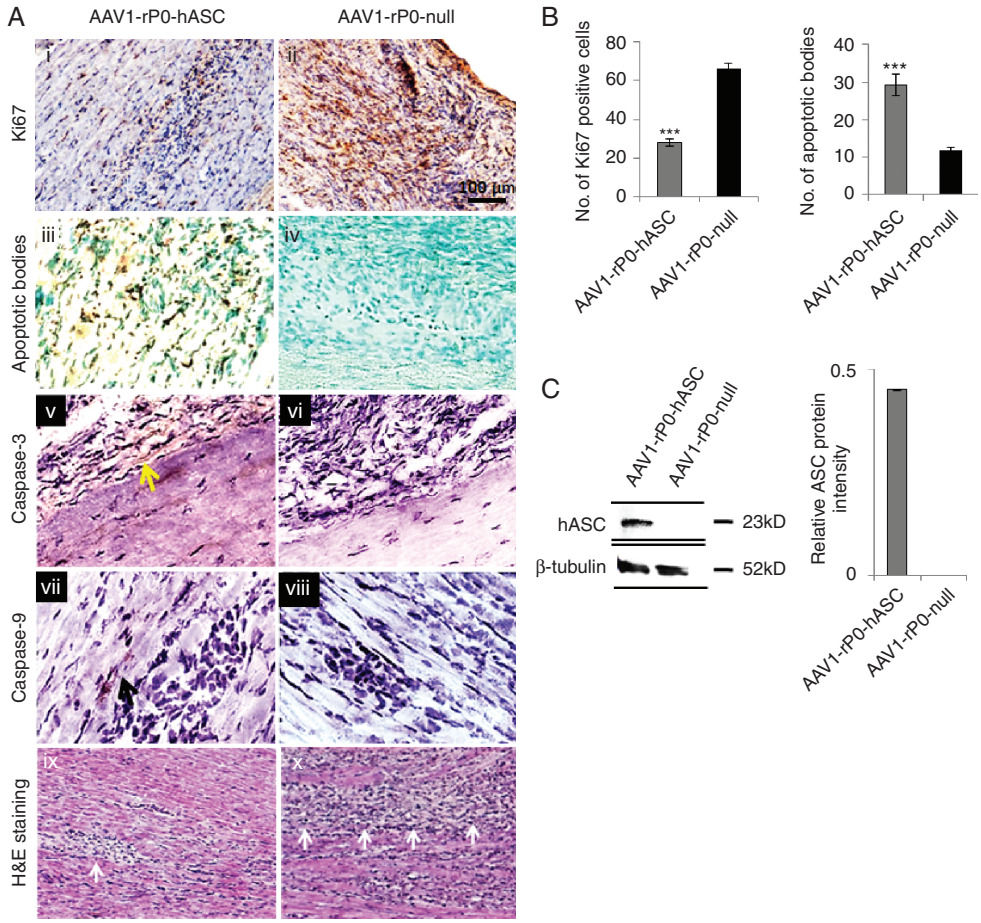

Fig. 6 AAV1-rP0-hASC in vivo therapy of HEI-193 human schwannoma increases markers of apoptosis and reduces markers of cell proliferation. (A) Immunostaining for the proliferation marker Ki67 in HEI-193 tumors injected with AAV1-rP0-hASC (i) or AAV1-rP0-null (brown stained, ii). Apoptosis staining of tumors injected with AAV1-rP0-hASC (brown stained, iii) or AAV1-rP0-null (iv). AAV1-rP0-hASC injected tumors showed elevated expression of caspase-3 (v) and caspase-9 (vii) in comparison to AAV1-rP0-null injected tumors (vi, viii). Hematoxylin and eosin (H&E) staining showed abundant, mitotically active hematoxylin-positive HEI-193 tumor cells (white arrows indicate dark-stained cells) after AAV1-rP0-null vector injections (x), but scant numbers of tumor cells after AAV1-rP0-hASC injection (ix). (B) Quantification of Ki67-positive cells and apoptotic bodies in the injected tumors. (C) Western blotting revealed ASC expression in the injected tumors (proteins extracted 1 wk post virus injection). Data are presented as means ± SEM ($n = 3$ per group). ***$P < 0.001$ (*t*-test).

In schwannoma tissue samples, we observed a mixed pattern of *ASC* methylation, most likely due to the presence of non-schwannoma cells, but purified primary vestibular sporadic schwannoma cells and HEI-193 cells indicate full *ASC* methylation, which further suggests that unmethylated cells found in tissues are likely to be non-schwannoma cells. Alternatively, some schwannomas may have incomplete methylation of the *ASC* promoter.

Our therapeutic approach utilizes intratumoral injection of an AAV1 vector for delivery of an *ASC* transgene under the control of the Schwann-cell specific promoter, P0. Neuronal protection is maintained by using the Schwann-lineage cell specific promoter, P0. Previous work from our lab demonstrated that there is essentially no expression of P0-controlled transgenes in sensory neurons,[9,34] but perhaps more importantly, neural protection is afforded by the fact that ASC is abundantly expressed in peripheral nerve, and therefore in sensory neurons. Based on our current data we further hypothesize that the physiologic expression of ASC in peripheral nerve prevents development of schwannomas by inducing apoptosis and, possibly, cell-cycle arrest.

Thus, we have identified ASC as a putative schwannoma tumor suppressor with high potential utility for schwannoma gene therapy and generated a therapeutic strategy that utilizes a novel mechanism that does not interfere or overlap with current schwannoma treatments. Translation of this AAV1-rP0-hASC schwannoma gene therapy to clinical application holds the promise of filling a major unmet clinical need for the individuals (and their families) who suffer from the constellation of genetic diseases that cause schwannomas to develop.

Supplementary Material

Supplementary data are available at *Neuro-Oncology* online.

Keywords

ASC | AAV1 | gene therapy | methylation | schwannoma

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