Cancer imaging and therapy utilizing a novel NIS-expressing adenovirus: The role of adenovirus death protein deletion

Matthew Glen Robertson,1 Benjamin Bruce Eidenschink,1,2 Eriko Iguchi,3,4 Stanislav O. Zakharkin,5 Christopher J. LaRocca,1 Ezequiel J. Tolosa,3 Mark J. Truty,6 Kari Jacobsen,1 Martin E. Fernandez-Zapico,3 and Julia Davydova1,7

Encoding the sodium iodide symporter (NIS) by an adenovirus (Ad) is a promising strategy to facilitate non-invasive imaging and radiotherapy of pancreatic cancer. However, insufficient levels of NIS expression in tumor cells have limited its clinical translation. To optimize Ad-based radiotherapy and imaging, we investigated the effect of Ad death protein (ADP) deletion on NIS expression. We cloned two sets of oncolytic NIS-expressing Ads that differed only in the presence or absence of ADP. We found that ADP expression negatively affected NIS membrane localization and inhibited radiotracer uptake. ADP deletion significantly improved NIS-based imaging in pancreatic cancer models including patient-derived xenografts, where effective imaging was possible for up to 6 weeks after a single virus injection. This study demonstrates that improved oncolysis may hinder the therapeutic effect of oncolytic viruses designed to express NIS. In vivo studies in combination with 131I showed potential for effective radiotherapy. This also highlights the need for further investigation into optimal timing of 131I administration and suggests that repeated doses of 131I should be considered to improve efficacy in clinical trials. We conclude that ADP deletion is essential for effective NIS-based theranostics in cancer.

INTRODUCTION

Oncolytic viral therapy continues to grow in importance as a cancer therapeutic strategy. In addition to direct cytolysis of tumor cells as part of viral replication and induction of tumor-specific immune responses, oncolytic viruses are also able to deliver transgenes to tumor cells. Early clinical trials with oncolytic viruses as single-agent therapies proved to be poorly effective. Consequently, inclusion of transgenes to selectively express therapeutic proteins has developed to increase the effectiveness of virotherapy.

The sodium iodide symporter (NIS) has become a popular candidate protein for insertion into oncolytic viruses.1 NIS is a native transmembrane glycoprotein primarily expressed in the thyroid that mediates iodine uptake. For many years, clinicians have exploited the function and limited native expression of NIS to facilitate radiiodine uptake, providing a unique imaging and treatment strategy for thyroid cancer.7 Not surprisingly, the idea to deliver NIS to other cancers using viral vectors has been extensively explored over the last decade, mainly for use as an adjunct cancer-imaging tool.1 Indeed, virus-mediated tumor expression of NIS can enable non-invasive tumor visualization through the use of standard SPECT/computed tomography (CT) or PET/CT imaging with commercially available radiotracers. This approach to deliver NIS for cancer imaging has been undertaken with many viral vectors, including adenovirus (Ad), measles, vaccinia, herpes simplex, poxvirus, and vesicular-stomatitis viruses.4

Over the last few years, our lab has designed oncolytic Ad (OAd) vectors that encode therapeutic proteins in the adenoviral E3 region (E3), which we refer to as the ΔE3 system.11–20 Many native genes from E3 are neither requisite for Ad infection or replication, and thus they can be deleted to provide space for transgenes while still preserving genome size and replication competence. Because transgene expression from E3 is controlled by the Ad major late promoter, therapeutic proteins are produced with each round of virus replication.11,15 In addition, we have shown that non-invasive detection of E3 transgenes can serve as a surrogate measure of virus replication to inform therapeutic potential.11

An important component of E3 is the Ad death protein (ADP), which promotes host cell membrane degradation to allow release of viral progeny.21,22 In our prior studies, we confirmed that deletion of the l2.5K,
6.7K, gp19K, RID-α and RID-β, and 14.7K genes, while maintaining ADP in the E3 region, results in overexpression of ADP and subsequent improvement in oncolysis.11 Subsequently, we have utilized our ΔE3 system to express various imaging and therapeutic transgenes (e.g., EGFP, RFP, luciferase, interferons, and NIS) in different tumor types.11,12,15 All of these constructs, including NIS-expressing OAd (OAd-NIS), were designed to overexpress ADP to improve virus spread and oncolysis in solid tumors. First generation ADP-positive (ADP(+)) OAd-NIS vectors demonstrated effective oncolysis and were able to mediate radioisotope uptake in prostate and lung cancer models.13,14,19 However, we were concerned that ADP may have deleterious effects on the membrane localization and functionality of NIS.

Here, we investigated the effect of ADP deletion on NIS expression and subsequent radiotracer uptake with the goal to improve NIS-based cancer therapy and imaging, focusing on pancreatic cancer. For this purpose, we cloned two sets of ΔE3-based OAd vectors expressing NIS from the E3 region that differ only in the presence or absence of ADP. Additional genetic modifications included utilization of a chimeric Ad5/Ad3 fiber to improve virus infectivity for coxsackie and Ad receptor (CAR)-deficient pancreatic ductal adenocarcinoma (PDAC) tumors and the cyclooxygenase-2 (COX2) promoter to drive tumor-selective replication.17,23,24 We compared NIS expression and radioiodine uptake in vitro and ex vivo and assessed virus-mediated in vivo imaging. We found that ADP deletion significantly improved NIS expression from E3, which resulted in improved imaging capabilities. We have also confirmed the feasibility of using our OAd-NIS vectors to facilitate radiotherapy with 131I.

RESULTS

NIS-expressing Ad structure
Four NIS-expressing Ad vectors were constructed resulting in two pairs of ADP(+) and ADP-negative (ADP(-)) replication-competent OAds with either wild-type (WT) or COX2-controlled replication (Figure 1A). The ADP gene was removed from ADP(-) vectors, while identical ADP(+) counterparts retained ADP in the E3 region. ADP status was confirmed using qPCR (Figure 1B).

Deletion of ADP does not affect the killing ability of OAd-NIS
The replication abilities and oncolytic potentials of NIS-expressing adenoviral vectors were evaluated in vitro with a crystal violet assay. The cancer cells were infected at a low titer of 1 viral particle (vp)/cell to allow at least a few rounds of virus replication (Figure 2A). At this low titer, ADP(-) viruses were slower to kill cells than their identical ADP(+) counterparts; however, their killing ability was similar to ADP(+) at later time points or upon higher viral titers (data not shown). Uniformly, vectors with COX2-regulated replication were less efficient at oncolysis than their WT-replication counterparts, but because these vectors also use the chimeric Ad5/3 fiber, their oncolytic potential remained comparable to Ad5WT. Importantly, COX2-controlled viruses demonstrated insignificant viral spread in COX2-/- BT474 cells, indicating the feasibility of the COX2 promoter to restrict novel OAd-NIS virus replication to COX-2+ cells consistent with our previous findings.17,23 To better quantify virus-induced cytolysis, we analyzed A549 and Panc-1 cell viability at low-titer infection using a cell proliferation assay (Figure 2B). Both ADP(+) and ADP(-) viruses produced a statistically significant (p < 0.05) decrease in cell viability compared to untreated controls. ADP(-) viruses were slower to kill than their ADP(+) counterparts (day 14 post-infection [p.i.] versus day 7 p.i.). These experiments demonstrate that despite being less efficient than ADP(+) counterparts in killing cancer cells in vitro, ADP-deleted viruses still achieve an appropriate oncolytic effect by the end of the experiment.

The COX2 promoter selectively limits OAd-NIS replication and radioiodine uptake ex vivo in human pancreatic cancer tissue slices
We tested the selectivity of NIS-expressing vectors through ex vivo infection of human pancreatic tumors and adjacent normal pancreatic tissues (Figure 3A). Viral copy numbers in tissues infected with the COX2 promoter-controlled virus (COX2-NIS) were seven orders of magnitude higher in tumor compared to normal pancreas (7 x 10^4 versus 2 x 10^4, respectively; Figure 3B). In contrast, WT-replication counterpart (WT-NIS)-infected tissues showed similar levels of virus replication in tumor and normal pancreas (1 x 10^4 versus 1 x 10^4, respectively). Of note, viral copies of COX2-NIS and WT-NIS in pancreatic tumor tissues were nearly equivalent (7 x 10^4 versus 1 x 10^4, respectively) in this preclinical model. Similarly, radioactivity after COX2-NIS
infection was significantly higher in tumors compared to normal tissue while iodine uptake in WT-NIS-infected tissues was not statistically different (Figure 3C).

**ADP deletion improves NIS expression in vitro**

To understand the impact of ADP on NIS expression, we performed immunofluorescent analyses and fluorescence-activated cell sorting (FACS) analyses of A549 cells infected with ADP(+) and ADP(−) vectors (Figures 4A and 4B). NIS expression was observed after infection with all NIS-expressing viruses, with significantly higher levels of NIS at later time points indicating replication-dependent expansion of gene expression. Immunostained cells clearly showed potentiated NIS expression with ADP(−) vectors compared to ADP(+) counterparts. These data correlated well with FACS analysis. Importantly, the ADP(−) Cox2-NIS-ADP(−) demonstrated clearly improved localization of NIS in cellular membrane when compared to its ADP(+) counterpart (Figures 4C and 4D). Overall, these analyses revealed higher levels of NIS expression and improved membrane localization of NIS after infection with ADP-deleted viruses.

**Feasibility of radioiodine therapy with NIS-expressing Ad vectors**

The ability of OAd-NIS to facilitate radiotherapy in vivo was evaluated in A549 subcutaneous tumors in mice (Figure 5A). Both oncolytic Cox2-NIS viruses (ADP(+) and ADP(−)) in combination with radioactive iodine (131I) showed statistically significant tumor reduction compared to the untreated control while the therapeutic effect of AdCMV-NIS combined with 131I was not significantly different. We also compared the growth curves of virus monotherapy versus combination therapies. While all viruses trended toward further restriction of tumor growth after combination with 131I therapy (compared to monotherapy groups), only the Cox2-NIS-ADP(+) treatment group reached statistical significance. Combination therapy with Cox2-NIS-ADP(+) was the most efficacious treatment regimen at killing tumor in this experiment. However, comparison of combination therapy with Cox2-NIS-ADP(−) versus Cox2-NIS-ADP(+) revealed no significant difference between these two groups.

11 and 19 days post treatment with Ad (8 and 16 days after administration), tumors and uninvolved leg muscle were measured for radioactivity using a gamma counter and tumor radioactivity was expressed relative to muscle (Figure 5B). Here we found correlation with the previous in vitro studies (Figure 4), with Cox2-NIS-ADP(−) outperforming its ADP(+) counterpart and AdCMV-NIS control. Cox2-NIS-ADP(−) infection resulted in radioactivity that was 2 times higher than PBS control (5.0 and 2.6, respectively). Tumors injected with Cox2-NIS-ADP(+) and AdCMV-NIS had radioactivity that was above control levels but lower than Cox2-NIS-ADP(−)-injected tumors (3.1 and 3.7, respectively). Radioactivity measurements had large variation between animals and consequently, the uptake differences did not achieve statistical significance. By day 19 p.i., the counts per minute (CPM) counts in tumors were not different from uninvolved leg muscles (data not shown). The nonsignificant differences in radiotracer uptake at day 11 and lack of radiotracer at day 19 could be due to complete clearance of 131I from the body, including tumors. These findings suggest that a single dose of 131I is likely insufficient to capitalize on the high levels and extended duration of Ad-mediated NIS expression. Furthermore, the absence of iodine at later time points could explain the limited therapeutic effect of the combination therapy with 131I.
ADP deletion enhances in vivo imaging capability of NIS-expressing vectors

The in vivo imaging potential of ADP(+) and ADP(-) NIS-expressing vectors was first compared in nude mice with human pancreatic cancer Panc1 xenografts. Two different doses of adenoviral vectors (high: \(4 \times 10^9\) and low: \(4 \times 10^8\) viral particles) were analyzed, with the lower dose being used to avoid a rapid virus-killing effect, to ensure radiotracer uptake could be evaluated (Figure 6A). At both dosing levels, SPECT/CT images showed much higher radiotracer uptake with Cox2-NIS-ADP(-) when compared to its ADP(+) counterpart and control vectors (WT-NIS-ADP(+) and AdCMV-NIS, a positive control employed in human clinical trials of NIS-mediated gene therapy for prostate cancer). In addition to improved uptake at each sampling, Cox2-NIS-ADP(-) facilitated tumor-specific \(^{99m}\)Tc uptake 4 weeks after a single virus injection compared to 1 week with AdCMV-NIS. The long duration of imaging capability indicates that NIS is being expressed in a replication-dependent manner, allowing for continued NIS production as the virus spreads throughout the tumor. In contrast, AdCMV-NIS is replication-deficient and thus unable to sustain NIS expression for extended periods of time. The functional advantage of replication-dependent gene expression for imaging is clearly visible in the results of this experiment.

Notably, while ADP deletion did not affect the spread of virus in solid tumors as shown by Ad-hexon staining, it did result in higher levels of NIS expression that remained robust for at least 4 weeks post-infection (Figure 6B). This accounts for the excellent duration of imaging capability of Cox2-NIS-ADP(-).

The therapeutic potential of the NIS-expressing vectors as monotherapies was also evaluated with two different viral doses (Figure 6C). All replication-competent NIS viruses showed statistically significant tumor reduction compared with untreated controls at both high and low viral titers. Of note, Cox2-NIS-ADP(+) and Cox2-NIS-ADP(-) were not statistically different at slowing tumor growth. The slower oncolysis with ADP(-) vectors seen in vitro did not appear to hamper the virus’s ability to control tumor growth in vivo. Overall, these data suggest that deletion of ADP has clear positive effects on the imaging capability of these OAd-NIS vectors and does not reduce their antitumor activity, compared to viruses expressing ADP.

ADP deletion improves NIS expression and subsequent imaging in PDAC patient-derived xenografts

Virus-mediated SPECT/CT imaging was next analyzed in PDAC patient-derived xenografts (PDAs). Similar to human disease, the human-biopsy-proven PDAC tissues (Figure S1) exhibited varied tumor growth rates leading to different lengths of time to reach euthanasia criteria and heterogeneous responses to virus treatment as measured by radiotracer uptake. To objectively evaluate viral effects across the heterogeneous PDX cohorts, after each imaging session, we quantified radiotracer uptake from the tumor of each mouse and compared it against an area of uninvolved muscle. This provided a relative difference (RD) in uptake between tumor and muscle for each animal at each time point, allowing for comparison across cohorts (Figure 7A). This analysis suggested significant improvement in radiotracer uptake in tumors injected with Cox2-NIS-ADP(-) compared with both Cox2-NIS-ADP(+) and AdCMV-NIS. Again, we observed excellent duration of imaging with ADP(-) that facilitated radiotracer uptake for up to 6 weeks after only a single viral injection. Illustrative images from two PDAC PDX cohorts (patients #177 and #27) are shown in Figure 7B.

We sought to characterize the PDX model and analyze viral spread and activity within patient tumors using immunohistochemical methods. Figure 7C shows sequential slices of a PDX infected with Cox2-NIS-ADP(-). Hematoxylin and eosin (H&E) staining shows evidence of cellular debris consistent with oncolysis (Figure 7C, image i). Collagen staining revealed a highly desmoplastic tumor, accurately reflecting the known microenvironment of human pancreatic cancer (Figure 7C, image ii). Immunohistochemistry (IHC) staining for Ad-hexon, NIS, and CYK19 (a marker of pancreatic ductal cells) show active adenoviral infection of pancreatic ductal cells within the tumor and virus-mediated NIS expression (Figure 7C, images iii–v). Active adenoviral infection as evidenced by Ad-hexon and NIS expression were present in tumors injected with either ADP(+) and ADP(-) viruses (Figure 7D). Ad-hexon expression quantified by ImageJ showed no statistical difference between the two tumor samples; however, NIS
expression was about 10-fold higher in tumors infected with Cox2-NIS-ADP(–) (Figure 7E).

We also measured tumor growth for PDX mice and plotted this data by treatment group (Figure 7F). As was seen with Panc1 xenografted mice, we observed a significant reduction in tumor growth upon injection of any NIS-expressing virus. No statistical difference was seen between the tumor burden of ADP(+) and ADP(–)-injected mice. All these data confirm active Ad infection and virus-induced NIS expression in human tumors infected with Cox2-NIS-ADP(–). Additionally, the data provide further support of Cox2-NIS-ADP(–) as a superior vector due to improved NIS expression leading to better imaging potential while also providing similar tumor control to Cox2-NIS-ADP(+).

DISCUSSION
For many decades, cancer treatment has benefitted from a multimodal approach that historically has included some combination of surgery, chemotherapy, and radiation with more recent additions of targeted biologics and immunotherapies. Oncolytic viruses designed to express the dual-function gene NIS offer the potential of a single theranostic agent that provides multiple clinical benefits including assisting in diagnosis and prognostication through improved cancer imaging, as well as combining direct oncolysis, tumor specific sensitization of host immunity, and radiotherapy. With such promise, we must ask why these viruses have thus far seen little use as theranostic agents. Indeed, while many NIS-expressing viruses have been reported as potential imaging tools in preclinical studies, few have been investigated for their ability to facilitate NIS-based radiotherapy.

Overall, the clinical translation of NIS-based imaging and radiotherapy remains a challenge and early clinical trials have focused mainly on monitoring virus spread. These studies have highlighted the difficulties in achieving sufficient NIS expression to support clinically relevant radiodine uptake in tumors.

The goal of this work was to remedy the problem of low NIS levels by optimizing the vectors to maximize NIS expression. In addition to NIS expression through the partially deleted E3 region, we utilized two previously established strategies to improve infectivity and selectivity in solid tumors including pancreatic adenocarcinoma. A chimeric fiber protein (Ad5/3) was used to promote virus infectivity in tumors by retargeting the virus to CD46 and desmoglein 2 receptors to overcome CAR deficiency on the surface of tumor cells. We also used the Cox2 promoter to drive Ad replication, limiting NIS expression and viral spread to Cox2+ tumors. These strategies functioned well in preclinical models where virus replication and NIS expression were effectively restricted to pancreatic tumors.

We chose to utilize our partial ΔE3 vector design because it achieves high levels of transgene expression and allows for monitoring of Ad replication. We were specifically interested in exploring the effect of the ADP on NIS expression from E3. More than a decade ago, Dornonin et al. demonstrated that deletion of non-essential E3 region genes while maintaining ADP resulted in higher levels of ADP expression and subsequently increased cytopathic effect. Since then, ADP-overexpressing vectors have been used by many, including us, with the goal to
improve the oncolytic potential of Ad-based therapeutic. However, for OAd-ΔE3-NIS vectors, our goal is not simply oncolysis, but also effective NIS expression, which we have now shown is enhanced by deletion of ADP. This work is the first to highlight the impacts of adenooviral ADP on NIS-facilitated radiotherapy and imaging. Our data suggest that ADP expression negatively affects NIS membrane localization thereby inhibiting radiotracer uptake. Moreover, ADP deletion improves transmembrane localization of NIS and allows the host cells to remain alive through more replication cycles, thus producing more functional NIS that can mediate radiotherapy and effective imaging in cancer. We have shown that ADP deletion significantly improves NIS-based imaging in different preclinical models including PDAC PDXs, thereby facilitating effective imaging for more than 6 weeks after only a single virus injection. Our experiments were performed with human tumors to assess replication-dependent NIS expression and killing ability of the oncolytic Ad; however, further investigations using immunocompetent models (e.g., Syrian hamsters) will be required to elucidate the host-virus interactions of our specific viral vector.

Effective radiotherapy facilitated by NIS-expressing oncolytic viruses continues to be difficult to achieve. Our radiotherapy experiment confirmed the possibility of OAd-ΔE3-NIS-mediated radiotherapy and paves the way to achieving true clinical benefit. Our imaging studies, in which technetium was administered each time immediately before SPECT/CT imaging, clearly show radiotracer uptake and vivid imaging in tumors for 4–6 weeks after a single injection of OAd-ΔE3-NIS. However, our radiotherapy experiments measured radiotherapy uptake 8 and 16 days after $^{131}$I injection and revealed lower uptake than would be expected based on hypothesized NIS levels extrapolated from in vitro and imaging experiments. Unlike thyroid tissue, which retains iodine in the cell by incorporating it into thyroid hormone, tumors lack this organification process and consequently $^{131}$I leaks out of tumor cells and is excreted.25 Once the initial $^{131}$I injection has been cleared from circulation, NIS expression can have no further therapeutic effect. We conclude that the limited improvement of combination therapy with $^{131}$I over virus monotherapy is due to the lack of circulating $^{131}$I for much of the duration of the experiment, and most of the observed restriction of tumor growth over the 27-day experiment was due to viral activity alone. Hence, we anticipate that repeated injections of $^{131}$I will be required for effective tumor destruction. Additionally, we anticipate that because of the heterogeneity seen in all cancers, the timing and dosage of $^{131}$I will vary and should be determined using non-invasive imaging techniques to verify adequate NIS expression. Standard dosimetry calculations based on radiotracer uptake seen by SPECT/CT imaging could provide a reasonable estimate of NIS activity in individual tumors and guide the start date and dosing of $^{131}$I therapy for maximal effect with minimal toxicity. The combination of non-invasive imaging with radiotherapy will likely prove to be a key component of effective oncolytic virus-mediated radiotherapy.

In conclusion, this study demonstrates that, counterintuitively, improving oncolysis may actually hinder the therapeutic effect of oncolytic viruses designed to express the NIS protein. By identifying the importance of ADP deletion to optimize functional NIS expression from the Ad E3 region, we have demonstrated the feasibility of radiotherapy and the superior imaging capabilities that can be achieved with NIS-expressing Ad in human pancreatic cancer models. Our findings also emphasize the importance of the timing of iodine administration to achieve clinically relevant results. We hope that this work will provide additional knowledge that can be applied in the design of future oncolytic viruses to facilitate NIS-based radiotherapy and imaging. Virus-mediated oncolysis combined with targeted $^{131}$I radiotherapy and improved imaging to both guide and monitor therapy has great potential to be transformative for devastating cancers such as pancreatic adenocarcinoma.

**MATERIALS AND METHODS**

**Cell lines**

The human PDAC Panc-1, ASPC1, MiaPaca2, lung carcinoma A549, and breast cancer BT474 cell lines were obtained from American Type Culture Collection. S2013 and S2VP10 cell lines representing the aggressive metastatic forms of PDAC were a generous gift from Dr. Selwyn Vickers.30,31 The cells were grown in DMEM supplemented with 1% penicillin streptomycin, 2 mM L-glutamine, and 5% fetal bovine serum (20% for ASPC1). BT474 was cultured in RPMI medium supplemented with 15% PBS and bovine insulin (0.01 mg/mL).

**Adenoviral vectors**

Two pairs of NIS-expressing ADP(+) and ADP(−) replication-competent OAds with WT and Cox2-controlled replication were

![Figure 5. Combination with $^{131}$I improved therapeutic effect of virotherapy](image-url)
constructed based on the previously reported E3 system. Briefly, the E3 3.6K and 7.5K genes were maintained while non-essential 6.7K, gp19K, RID-α, and -β, 14.7K were deleted.11,15,16 The initiating ATG codon of the 12.5 gene was inactivated by replacement with TTA.15 The ADP gene was removed from ADP(–) vectors, while identical ADP(+) counterparts retained ADP in the E3 region. The viruses were propagated in A549 cells, purified by cesium-chloride gradient ultracentrifugation, and dialyzed in PBS with 10% glycerol. Titration was performed with a plaque-forming unit (PFU) assay and optical density-based measurements.32 The vp/ PFU ratios for these vectors were in the range of 10–80. Purified virions were confirmed by qPCR to contain the Cox2 promoter, 5/3 fiber, NIS, and ADP. As a control vector, replication-deficient AdCMV-NIS employed in the phase I clinical trial of NIS-based therapy for men with prostate cancer (ClinicalTrials.gov: NCT00788307) was used.3

Crystal violet assay
2.5 × 10⁴ cells per well were plated in 12-well plates and infected at 1–10 vp/cell in 200 µL of appropriate medium. 3 h p.i., medium was replaced and incubated up to 12 days. Medium was aspirated and cells fixed with 10% buffered formalin followed with 1% crystal violet staining.23

Cell viability assay
3,000 cells per well were plated in 96-well plates and infected at 10 vp/cell in 100 µL of appropriate medium. On the next day, 100 µL of the growth medium containing 1% FBS was added. The cells were incubated for 5–12 days and the number of viable cells was determined using the Cell Titer 96 Aqueous Cell Proliferation Assay as instructed by the manufacturer. Absorbance was measured at a wavelength of 490 nm. Data are expressed as percent viability relative to untreated control cells (set at 100%). Experiments were performed in triplicate and averaged.

Ex vivo studies in human specimens
Remnant de-identified specimens of human PDAC and adjacent normal pancreas were obtained following surgical resection and were immediately sliced with the Krumdieck tissue slicer.17 Tissue slices 200 µm thick were plated in 12-well plates containing 50:50 Ham’s/F12 and DMEM medium supplemented with 1% penicillin/streptomycin, 1% amphotericin B, 15% FBS, and 10 µg/mL dexamethasone. The average tissue weights were used to calculate the approximate cell number per tissue slice and infection was subsequently performed at 100 vp/cell. Infection media was replaced with fresh culture media 3 h p.i. At day 2, the total DNA was extracted from the slices using a QIAamp DNA Blood Mini kit. Viral copy number was quantitated by SYBR Green RT-PCR with adenoviral E4 primers and compensated with β-actin.23,33

Iodine-125 uptake
Human PDAC and adjacent normal pancreas tissues were seeded in 12-well plates and infected with 1 vp/cell. Culture media was aspirated
2 days p.i. and replaced with 10 mM HEPES in HSBS containing 0.1 mM NaI. Control incubations included 0.1 mM KCLO4 (NIS inhibitor) to confirm NIS-specific radioiodine uptake. Cells were incubated for 45 min with 105 CPM of 125I, followed by aspiration and rinsing in ice-cold Hank’s balanced salt solution, then lysed in 1 M NaOH for 5 min with shaking. Radioactivity of lysates was measured using a gamma counter.19

**Immunofluorescent analyses and histologic analysis**

1. **Cell line preparation:** cells were grown on chamber slides (50,000 per well), treated with 10 vp/cell in 100 μL of growth medium and incubated. Cells were washed in PBS, fixed (4% parafomaldehyde: 20 min; ice), and permeabilized (0.05% Triton X-100; 5 min; ice).

2. **Tissue preparation:** PDAC Panc1 and PDX tumors were paraffin embedded, sectioned, and processed for antibody staining. Antigen retrieval was performed using a 1X universal antigen retrieval solution (R&D Systems, Minneapolis, MN, USA). Tissue sections were permeabilized (0.05% Triton X-100; 5 min; ice) and then incubated in a 5% BSA solution for 1 h at room temperature to reduce background.

3. **Immune staining:** fixed and permeabilized cells and tissues were incubated on ice for 1 h (cells) or overnight (tissue) in a 5% (cells) or 2% (tissues) BSA solution containing combinations of the following primary antibodies at indicated dilution: anti-NIS (anti-FP5A, 1:500), anti-cytoskeleton associated protein (CYK4, 1:300), anti-cytokeratin-19 (RCK 108, 1:300), and anti-hexon-fluo-rescein isothiocyanate (FITC; AB1056, Millipore; 1:1,000). Cells and tissues were then incubated at room temperature for 30 min in 5% BSA (cells) or 1 h in 2% BSA (tissues) solutions containing Phycoerythrin (PE)-conjugated (1:1,000; red) and/or
tumor volume = \(\text{width}^2\) measured twice per week with calipers and was calculated as follows:

\(\text{tumor volume} = \frac{\text{length}}{2}\).

Biomedical Image Quanti
er. Intratumoral radioisotope uptake was analyzed using PMOD
SPECT/CT images were acquired 1 h later using a U-SPECT II scan-
xenografts (n = 8/group) at 6
50 injection of 0.5 mCi of sodium pertechnetate (99mTcO4-), and
continued for 10 days after131I injection. 3 days p.i., 3.0 mCi of phar-
was supplemented with T4 (5 mg/L) 2 weeks prior to iodine admin-
were described above. Three groups received Ad monotherapy, three
Two groups served as control (PBS alone and131I alone). Established
glial adenovirus (OAd-NIS) vectors were evaluated with general
mean, and 16 days after131I administration), tumors and leg muscles were
uptake to tissue weight (CPM/mg) for tumors \((n = 8)\) and then
dividing by the corresponding CPM/mg for leg muscle of the same
mouse. Tumor volume was calculated as described above. All animal
studies were approved by institutional review boards and carried out
in accordance with established animal care protocols.

Flow cytometry
Untreated or virus-treated cells (10 vp/cell for 3 h) were sub-cultured
in growth medium, washed in PBS, fixed while maintaining a single-
cell suspension (4% paraformaldehyde: 20 min; ice), and washed
again in PBS before permeabilization (0.05% Triton X-100; 5 min;
ice). Cells were incubated in a 5% BSA solution containing anti-NIS
primary antibody (FP5A; 1:500) for 30 min on ice followed by PE-
conjugated secondary antibody (1:1,000) and incubated for 30 min
at room temperature. Cells were washed and re-suspended in PBS
before analysis using a FacsCanto.

Non-invasive imaging and tumor control in vivo tumor models
To establish tumor models, we used: (1) pancreatic cancer Panc1 cells
\(2 \times 10^6\) per injection site); and (2) PDX derived from 6 human pa-
tients with biopsy-proven PDAC (Figure S1). The cells or tissues were
inoculated subcutaneously into the right flank of female nude mice
(6–8 weeks, Frederick Cancer Research). The tumor nodules were in-
jected with a single virus dose when they reached a size of 8–10 mm in
diameter \(4 \times 10^9\) or \(4 \times 10^{10}\) vp/tumor in Panc1 models, and \(4 \times
10^{10}\) vp/tumor in PDX models; mixed with PBS for total volume of
50 μL. A high-resolution micro-SPECT/CT system was used for
planar and fusion images as we described previously. Briefly, 2
days p.i., and twice per week thereafter, mice received intraperito-
eal injections of 0.5 mCi of sodium pertechnetate \((^{99m}\text{TeO}_4^-)\), and
SPECT/CT images were acquired 1 h later using a U-SPECT II scan-
er. Intratumoral radioisotope uptake was analyzed using PMOD
Biomedical Image Quantification Software. Tumor volume was
measured twice per week with calipers and was calculated as follows:
tumor volume = \((\text{width}^2 \times \text{length})/2\).

In vivo therapeutic studies in combination with radioactive
iodine \((^{131}\text{I})\)
A549 cells (2.4 \times 10^6) were injected subcutaneously into nude mice as
we described above. Three groups received Ad monotherapy, three
other groups received combination therapies (Ad plus \(^{131}I\)), and
two groups served as control (PBS alone and \(^{131}I\) alone). Established
 xenografts \((n = 8/group)\) at 6–8 mm in diameter were injected with
50 μL of either PBS or Ad at \(4 \times 10^{10}\) vp/tumor. Water for animals
was supplemented with T4 (5 mg/L) 2 weeks prior to iodine admin-
istration and continued for the entire study. Low-iodine diet was pre-
scribed to all animals 10 days before iodine administration and
continued for 10 days after \(^{131}I\) injection. 3 days p.i., 3.0 mCi of phar-
aceutical grade \(^{131}I\) (Cardinal Health, delivered in pre-dosed sy-
ringes) were injected intraperitoneally in the hood equipped with
Hepa and charcoal filters. The dose range was calculated based on
FDA recommended body surface area conversion to provide doses
comparable to those in humans. 11 and 19 days p.i. with Ad (8
and 16 days after \(^{131}I\) administration), tumors and leg muscles were
harvested and \(^{131}I\) uptake was determined using a gamma counter.
Virus-induced \(^{131}I\) uptake was determined by first adjusting the \(^{131}I\)
uptake to tissue weight (CPM/mg) for tumors \((n = 8)\) and then
dividing by the corresponding CPM/mg for leg muscle of the same
mouse. Tumor volume was calculated as described above. All animal
studies were approved by institutional review boards and carried out
in accordance with established animal care protocols.

Statistical analysis
Statistical analyses were done with R 3.4.4. Differences in viral copy
numbers between tumor and normal pancreatic tissues were evalu-
ated using a general linear model. Analysis of iodine intake data
was done using mixed models in R package lme4 with the viral
construct treated as a fixed effect and the mouse as a random effect.
The differences in dynamics of tumor growth between treatment
groups were evaluated using repeated-measures analysis with R pack-
age nlme. The autoregressive correlation structure of the first order
corCAR1) was to account for correlation between adjustment time
points. The viral constructs, week, and their interaction were treated
as fixed effects and mouse as a random effect. The differences in on-
colytic potential of OAd-NIS vectors were evaluated with general
linear models using Day, Construct, and their interaction as predic-
tors. For all analyses, pairwise comparisons between groups were
done using R package emmeans with Tukey’s adjustment. Remaining
statistical analyses done with a two-tailed t test performed in Micro-
soft Excel. p values <0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.03.002.

ACKNOWLEDGMENTS
This study was supported by NIH NCI R01CA174861 (J.D. and
M.E.F.-Z.); NIH NCI R01CA228760 (J.D.); NIH NCI
P50CA101955 UAB-UMN Pancreatic Cancer SPOR Career Devel-
opment Award (J.D.); University of Minnesota Masonic Cancer Cen-
ter CRTI Translational Research Award grant (J.D. and M.G.R.); and
Randy Shaver Cancer Research Grant (J.D. and M.G.R.). The authors
thank Drs. John Morris (Mayo Clinic) and Lisa Koodie (UMN) for
valuable discussions and technical support.

AUTHOR CONTRIBUTIONS
Conceptualization, J.D. and M.E.F.-Z.; methodology, B.B.E., E.I., and
M.E.F.-Z.; investigation, M.G.R., B.B.E., E.I., E.J.T., K.J., and J.D.; re-
sources, M.J.T.; formal analysis, E.I. and S.O.Z.; writing – original
draft, M.G.R. and J.D.; writing – review & editing, M.G.R., C.J.L.,
M.E.F.-Z., and J.D.; funding acquisition, M.G.R., M.E.F.-Z., and
J.D.; supervision, M.E.F.-Z. and J.D.

DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES

1. Miller, A., and Russell, S.J. (2016). The use of the NIS reporter gene for optimizing oncolytic virotherapy. Expert Opin. Biol. Ther. 16, 15–32.

2. Tsuchefuy, Y., Franken, P., and Harrington, K.J. (2012). Radiovirotherapy: principles and prospects in oncology. Curr. Pharm. Des. 18, 3313–3320.

3. Dwyer, R.M., Schatz, S.M., Bergert, E.R., Myers, R.M., Harvey, M.E., Classic, K.L., Blanco, M.C., Frisk, C.S., Marler, R.J., Davis, B.J., et al. (2005). A preclinical large animal model of adenovirus-mediated expression of the sodium-iodide symporter for radioiodide imaging and therapy of locally recurrent prostate cancer. Mol. Ther. 12, 835–841.

4. Dingli, D., Peng, K.W., Harvey, M.E., Greipp, P.R., O’Connor, M.K., Cattaneo, R., Morris, J.C., and Russell, S.J. (2004). Image-guided radiovirotherapy for multiple myeloma using a recombinant measles virus expressing the thyroidal sodium iodide symporter. Blood 103, 1641–1646.

5. Haddad, D., Chen, N.G., Zhang, Q., Chen, C.H., Yu, Y.A., Gonzalez, L., Carpenter, S.G., Carson, J., Xu, J., Mittra, A., et al. (2011). Insertion of the human sodium iodide symporter to facilitate deep tissue imaging does not alter oncolytic or replication capability of a novel vaccinia virus. J. Transl. Med. 9, 36.

6. Li, H., Nakashima, H., Decklever, T.D., Nace, R.A., and Russell, S.J. (2013). HSV-NIS, an oncolytic herpes simplex virus type 1 encoding human sodium iodide symporter for preclinical prostate cancer radiovirotherapy. Cancer Gene Ther. 20, 478–485.

7. Naik, S., Nace, R., Federspiel, M.J., Barber, G.N., Peng, K.W., and Russell, S.J. (2012). Curative one-shot systemic virotherapy in murine myeloma. Leukemia 26, 1870–1878.

8. Rajecki, M., Sarparanta, M., Hakkarainen, T., Tenhunen, M., Diaconu, I., Kuhmonen, V., Kairemo, K., Kanerva, A., Airaksinen, A.J., and Hemminki, A. (2012). SPECT/CT imaging of hNIS expression after intravenous delivery of an oncolytic adenovirus and 131I. PLoS ONE 7, e23871.

9. Spitzweg, C., Dietz, A.B., Rajecki, M.K., Bergert, E.R., Tindall, D.J., Young, C.Y., and Morris, J.C. (2001). In vivo sodium iodide symporter gene therapy for prostate cancer. Gene Ther. 8, 1524–1531.

10. Warner, S.G., Kim, S.I., Chaurasiya, S., O’Leary, M.P., Lu, J., Sivanandam, V., Woo, Y., Chen, N.G., and Fong, Y. (2019). A Novel Chimeric Poxvirus Encoding hNIS Is Tumor-Tropic, Imageable, and Synergistic with Radioiodine to Sustain Colon Cancer Regression. Mol. Ther. Oncolytics 13, 82–92.

11. Dasydova, J., Gavrikova, T., Brown, E.I., Luo, X., Curiel, D.T., Vickers, S.M., and Yamamoto, M. (2010). In vivo bioimaging tracks conditionally replicating adenoviral infection and provides an early indication of viral antitumor efficacy. Cancer Sci. 101, 474–481.

12. LaRocca, C.J., Han, J., Gavrikova, T., Armstrong, L., Oliveira, A.R., Shanley, R., Vickers, S.M., Yamamoto, M., and Dasydova, J. (2015). Oncolytic adenovirus expressing interferon alpha in a syngeneic Syrian hamster model for the treatment of pancreatic cancer. Surgery 157, 888–898.

13. Oneil, M.J., Trujillo, M.A., Davydova, J., McDonough, S., Yamamoto, M., and Morris, J.C. (2013). Effect of increased viral replication and infectivity enhancement on radioidine uptake and oncolytic activity of adenovirus vectors expressing the sodium iodide symporter. Cancer Gene Ther. 20, 195–200.

14. Oneil, M.J., Trujillo, M.A., Davydova, J., McDonough, S., Yamamoto, M., and Morris, J.C. (2012). Characterization of infectivity-enhanced conditionally replicating adenovectors for prostate cancer radiovirotherapy. Hum. Gene Ther. 23, 951–961.

15. Ono, H.A., Le, L.P., Davydova, J.G., Gavrikova, T., and Yamamoto, M. (2005). Noninvasive visualization of adenovirus replication with a fluorescent reporter in the E3 region. Cancer Res. 65, 10154–10158.

16. Salvesed, A.O., Han, J., LaRocca, C.J., Shanley, R., Yamamoto, M., and Davydova, J. (2018). Combination of interferon-expressing oncolytic adenovirus with chemotherap and radiation is highly synergistic in hamster model of pancreatic cancer. Oncotarget 9, 18041–18052.

17. Armstrong, L., Arrington, A., Han, J., Gavrikova, T., Brown, E., Yamamoto, M., Vickers, S.M., and Davydova, J. (2012). Generation of a novel, cyclooxygenase-2-targeted, interferon-expressing, conditionally replicative adenovirus for pancreatic cancer therapy. Am. J. Surg. 204, 741–750.