Detection of neuroendocrine tumors using promoter-specific secreted *Gaussia* luciferase

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Abstract. Accurate detection of neuroendocrine (NE) tumors is critically important for better prognosis and treatment outcomes in patients. To demonstrate the efficacy of using an adenoviral vector for the detection of NE tumors, we have constructed a pair of adenoviral vectors which, in combination, can conditionally replicate and release *Gaussia* luciferase into the circulation after infecting the NE tumors. The expression of these two vectors is regulated upstream by an INSM1-promoter (insulinoma-associated-1) that is specifically active in NE tumors and developing NE tissues, but silenced in normal adult tissues. In order to retain the tumor-specificity of the INSM1 promoter, we have modified the promoter using the core insulator sequence from the chicken β-globin HS4 insulator and the neuronal restrictive silencing element (NRSE). This modified INSM1-promoter can retain NE tumor specificity in an adenoviral construct while driving a mutated adenovirus *E1A* gene (*Δ24E1A*), the *Metridia*, or *Gaussia* luciferase gene. The *in vitro* cell line and mouse xenograft human tumor studies revealed the NE specificity of the INSM1-promoter in NE lung cancer, neuroblastoma, medulloblastoma, retinoblastoma, and insulinoma. When we combined the INSM1-promoter driven *Gaussia* luciferase with *Δ24E1A*, the co-infected NE tumor secreted higher levels of *Gaussia* luciferase as compared to the INSM1p-*Gaussia* virus alone. In a mouse subcutaneous xenograft tumor model, the combination viruses secreted detectable level of *Gaussia* luciferase after infecting an INSM1-positive NE lung tumor for ≥12 days. Therefore, the INSM1-promoter specific conditional replicating adenovirus represents a sensitive diagnostic tool to aid clinicians in the detection of NE tumors.

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Abbreviations: NE, neuroendocrine; INSM1, insulinoma-associated-1; NRSE, neuronal restrictive silencing element; *Luc2*, luciferase2; Gau, Gaussia; Met, Metridia; MOI, multiplicity of infection; RFU, reflective fluorescence unit

Key words: adenovirus, detection, *Gaussia*, insulinoma-associated-1, neuroendocrine tumor

Introduction

Neuroendocrine (NE) carcinomas are rare neoplasms that can develop into highly malignant and life-threatening tumors (1,2). While they share a number of genetic and phenotypic traits, NE carcinomas comprise a very heterogeneous population of tumor types that can arise in various organs throughout the body. The most common of these cancers include neuroblastomas, retinoblastomas, medulloblastomas, pituitary carcinomas, small cell lung carcinomas, and carcinoid tumors, encompassing a broad spectrum of tumors that have so far required multiple detection and treatment methods (3-7). Despite their differences, many of these tumors express common tumor-specific markers that can identify them as NE cancers (8,9). Consequently, early detection of these tumor markers can lead to better treatment response and outcomes. The *INSM1* gene encodes a NE tumor-specific marker that was discovered using an insulinoma subtractive hybridization screen (10,11). The INSM1-promoter regulates the expression of INSM1, a transcription factor with a zinc-finger DNA binding domain that is highly specific for NE tumors (12). Through an *Insm1* knockout mouse model, Insm1 transcription factor was found to be important in the formation of endocrine pancreas and sympatho-adrenal lineage during development (13,14). Most interestingly, INSM1 expression was discovered to be restricted to the embryonic peripheral and central nervous system, specifically in the cells of neuroendocrine origin (15). The expression pattern was detected in the embryonic tissues of pituitary, pancreas, stomach, duodenum, thymus, adrenal glands, brain, and spinal cord, which were all found to be Insm1-positive at E15.5 in mice (16,17). However, INSM1 is silenced in normal adult tissues, but reactivated in most of the human NE tumors, including neuroblastoma, medulloblastoma, pheochromocytoma, small cell lung carcinoma,

insulinomas, pituitary tumors, carcinoid tumors, medullary thyroid carcinoma, and retinoblastoma (18). Therefore, INSM1 is a NE-specific tumor marker.

In order to assist with the detection of NE tumors despite their heterogeneous population, we have taken advantage of the INSM1-promoter's specificity in NE tumors to drive the expression of a downstream *Gaussia* luciferase gene. Secreted luciferases like *Metridia* or *Gaussia* luciferase have been shown to be highly luminescent, exhibiting 2-4-fold higher signal than *Renilla* or *firefly* luciferases (19,20). We have constructed INSM1p-*Met* and INSM1p-*Gau* reporter vectors to measure the INSM1 promoter activity in NE tumors. *In vitro* cell lines and xenograft human tumor cultured cells revealed positive luciferase secreted from NE tumors. In addition, combining the INSM1p-*Δ24E1A* and INSM1p-*Gau* luciferase vectors increased the sensitivity of secreted *Gaussia in vivo*. The *Δ24E1A* gene, a mutant form of the adenovirus *E1A* gene with a 24-bp deletion, is inactive in retinoblastoma (Rb) protein expressing cells and active in Rb-negative cancer cells (21). The cancer specificity from the modified INSM1 promoter and the *Δ24E1A* gene create a dual layer of safety against non-specific expression.

Materials and methods

Construction of adenoviral vectors. The Ad-INSM1p-*Met* construct was cloned using an original pGL3-INSM1p vector that contained the modified INSM1-promoter with HS4 insulator upstream and 2X NRSE downstream (22). The *Metridia* luciferase gene was excised from the pMet-Reporter vector (Clontech, Mountain View, CA, USA) and ligated downstream of the modified INSM1-promoter in pGL3. The pGL3 vector was cut to release the INSM1p-*Met* fragment, which was then ligated into the pShuttle plasmid (Agilent Technologies, Santa Clara, CA, USA) for adenoviral vector. The Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* constructs were cloned using the modified INSM1-promoter on the pGL3-INSM1p vector, created by shortening the full insulator sequence into two copies of the core HS4 insulator. The *Gaussia* luciferase gene was obtained from the pMCS-*Gaussia*-Dura Luc vector (Thermo Fisher Scientific, Waltham, MA, USA) and ligated downstream of the INSM1-promoter to create pGL3-INSM1p-*Gau*. To clone the *Δ24E1A* gene, site directed mutagenesis was performed on an existing *E1A* gene in the pJet plasmid (Thermo Fisher Scientific) to delete 24 bp from the original sequence. This *Δ24E1A* gene was then cloned into the pGL3 vector to form pGL3-INSM1p-*Δ24E1A*. Both the INSM1p-*Gau* and the INSM1p-*Δ24E1A* fragments were excised from their vectors and placed into the pShuttle plasmid. The Ad-SV40-*Luc2* construct was generated by excising the SV40 promoter from the pSEAP2-Control vector (Clontech) and ligated upstream of the *Luc2* reporter gene in the pGL4.10 vector (Promega, Madison, WI, USA). The SV40-*Luc2* fragment was cloned into the pShuttle vector. The pShuttle plasmid was linearized and electroporated into BJ5183-AD-1 cells (Agilent Technologies) to undergo recombination. After selection for the recombinants, linear adenoviral DNA was transfected into AD293 cells (Agilent Technologies) using FuGENE 6 reagent (Promega). The virus was amplified onto forty 150-mm tissue culture dishes and purified by CsCl gradient. This purified virus was then titered using the Adeno-X-Rapid Titer kit (Clontech, Mountain View) and stored at -80˚C. All sequences in the cloning process were verified through DNA sequencing.

In vivo luciferase imaging. Nu/Nu mice (National Cancer Institute, Bethesda, MD, USA), aged 8-10 weeks, received intravenous tail vein injection of either the modified first generation Ad-INSM1p-*Luc2*, the second generation Ad-INSM1p-*Luc2*, or unmodified Ad-INSM1p-*Luc2*. The viruses were prepared in phosphate-buffered saline at a concentration of 10^{10} ifu/ml and 100 μl of the viral solution was delivered slowly into the tail vein via a 27-gauge needle. To perform the imaging analysis for luciferase activity, D-luciferin substrate (Biosynth, Itasca, IL, USA) was prepared at a concentration of 15 mg/ml and injected intraperitoneally into mice at a dose of 150 mg/kg. Once injection was completed, the mice were anesthetized in an isofluorane chamber (2-4% by inhalation) before being transferred to a Kodak In-Vivo Multispectral FX imager (Carestream Health, Rochester, NY, USA). Using the imager's software, luminescence was acquired with a 10-min exposure and an X-ray image of the mice in the same position was acquired with a 2-min exposure. Imaging was performed 48 h after virus injection and periodically for ≤28 days. To generate the complete image, the luminescence acquisition was converted into a rainbow intensity scale and superimposed onto the X-ray acquisition using ImageJ software (National Institutes of Health, Bethesda MD, USA). For the NE tumor imaging, H1155 NE lung tumor cells ($1x10^7$) were pre-infected with the second generation modified Ad-INSM1p-*Luc2* virus (50 MOI) for 24 h and injected subcutaneously into the right hind flank of nude mice (n=3). After one week, the tumor growth was evidenced and imaged to show the modified INSM1 promoter specificity.

In vitro Metridia and Gaussia luciferase secretion assay. Cells were seeded in a 96-well plate at a density of 10,000 cells per well. After incubation at 37˚C and 5% CO_2 for 1 h, cells were infected with either no virus (negative control), Ad-INSM1p-*Met* (0-50 MOI), Ad-INSM1p-*Gau* (0-50 MOI), or Ad-SV40-*Luc2* (5 MOI). Infected cells were then incubated at 37˚C, 5% CO_2 for 24 h. After incubation, each well was washed gently with 1X PBS and replaced with fresh media for another 24 h. Fifty microliters of media per well were transferred to a 96-well white microplate. Luminescence was detected using the Pierce *Gaussia* Luciferase Glow Assay kit (Thermo Fisher Scientific), and read on a TopCount NXT Microplate Scintillation and Luminescence Counter. The adenoviral infection efficiency was determined by normalization (ratio) with intracellular luciferase (Ad-SV40-*Luc2*) using the Dual-Glo luciferase assay system (Promega) and read on a TopCount NXT Microplate Scintillation and Luminescence counter. To test the effects of Ad-INSM1p-*Gau* in combination with the Ad-INSM1p-*Δ24E1A* conditionally replicating adenovirus, cells were infected with a combination of 10 MOI Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* for a total of 20 MOI ($2x10^5$ ifu). Media was collected 2, 4, and 6 days (20 μl each day) after infection to determine secreted luciferase activity. Luminescence was detected using the Pierce *Gaussia* Luciferase Glow Assay kit (Thermo Fisher Scientific),

then read on a TopCount NXT Microplate Scintillation and Luminescence Counter. The Student's t-test with a threshold of $p<0.05$ was used to determine statistical significance. This process was repeated with the Ad-INSM1p-*Gau* infected cells to determine the Ad-INSM1p-*Gau*/Ad-SV40-*Luc2* ratio.

Xenograft human tumor culture assay. Xenograft tumors were prepared by injecting human tumor cells ($1x10^7$), such as HeLa, U87, D283, UMC-11, SK-NBe(2), H1155, and H69 subcutaneously into the right hind flank of nude mice. Tumor tissues were harvested and frozen (-80˚C) in RPMI-1640 culture medium with 10% DMSO. The cultured tumor cells were prepared by rapidly thawing in a 37˚C water bath and subsequent mincing into small sections ~1 mm^3 in size. The minced tissues were then centrifuged at 250 x g for 1 min and incubated in 2.5% trypsin for a total of 30 min at 37˚C and 2 ml growth media was added to neutralize the trypsin. The trypsinized tissues were then filtered through a 70-μm sieve and centrifuged again at 250 x g for 5 min. The 96-well clear-bottom plates were coated with 75 μl per well of a 1:6 dilution of Matrigel in RPMI growth media and then incubated for 30 min at 37˚C. The tumor cells (10,000 cells) were re-suspended in RPMI media and added to each Matrigel coated well. Cells from each tumor were infected with *Metridia*-luciferase and Ad-SV40-*Luc2* at 37˚C for 24 h. The ratio of *Metridia*/*Luc2* luciferase was calculated and averaged using RFU from 50 μl media per well.

Detection of serum Gaussia luciferase in vivo. Eight-week-old Nu/Nu mice (National Cancer Institute) were injected with H1155 NE lung tumor cells ($1x10^7$) subcutaneously into the right hind flank. Tumors were allowed to establish until tumor size grew to ≥0.1 cm^3 in volume. The mice were injected intra-tumorally with $1x10^9$ ifu of Ad-INSM1p-*Luc2* virus, or a combination of $5x10^8$ ifu of Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* (for a total of $1x10^9$ ifu). To detect the *Gaussia* expression in the bloodstream, 100 μl of blood was drawn at 3, 6, 9, and 12 days after virus injection. All animal experiments were performed in accordance with the approved protocol from the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center New Orleans. The collected blood was allowed to clot for 30 min at room temperature and centrifuged at 2,000 g for 10 min. Serum was collected from the supernatant and diluted with PBS at a 1:10 ratio. To detect *Gaussia* luciferase in the serum, 50 μl of the diluted serum from each sample was added to a flat bottom 96-well plate for the *Gaussia* luciferase assay.

Statistical analysis. Values were corrected and expressed relative to a control group. All experiments were repeated three times. Results are presented as mean ± SEM. Statistical analysis was performed using wither the Student's t-test when only two groups were in the experiment or by an one-way ANOVA comparison of multiple groups using the Tukey-Kramer test with differences at p-value of <0.05 being considered significant.

Results

Cloning the INSM1-promoter driven adenoviral constructs. To generate an adenoviral vector that is useful for the diagnosis of INSM1-positive NE tumors, we constructed the first generation modified INSM1-promoter by inserting a full HS4 insulator sequence upstream of the INSM1-promoter (~1.7 kb) along with two NRSE enhancer sequences in tandem repeats downstream and *Luc2* gene (Fig. 1A) (22). The modified INSM1-promoter drives a downstream *Metridia* luciferase gene, resulting in the construct Ad-HS4ins-INSM1p-2xNRSE-*Metridia* (Ad-INSM1p-*Met*) (Fig. 1B). A second generation of the modified INSM1-promoter was constructed to drive the expression of *Gaussia* luciferase and *Δ24E1A*. This promoter was created using two copies of the HS4 core insulator in place of the full insulator sequence. The final constructs Ad-2xHS4Core-INSM1p-2xNRSE-*Gaussia* (Ad-INSM1p-*Gau*) and Ad-2xHS4Core-INSM1p-2xNRSE-*Δ24E1A* (Ad-INSM1p-*Δ24E1A*), have a modified INSM1-promoter that is ~700 bp shorter than that of the promoter in Ad-INSM1p-*Met* (Fig. 1C and D). Ad-SV40-*Luc2* vector was constructed as a control vector (Fig. 1E).

We tested whether an adenoviral vector driven by the modified INSM1-promoter would result in non-specific expression *in vivo*. Tail vein injection was performed using three viral vectors, the unmodified Ad-INSM1p-*Luc2* (Fig. 1F), the first generation of the modified Ad-INSM1p-*Luc2* (Fig. 1G), and the second generation modified Ad-INSMp-*Luc2* (Fig. 1H) injected into non-tumor bearing Nu/Nu mice separately. Therefore, we examined the INSM1 promoter specificity with or without HS4 insulator and NRSE enhancer sequence. After a period of 2-28 days, luciferase activity was determined via *in vivo* imaging system after intraperitoneal (i.p.) injection of luciferin substrate using a Kodak In-Vivo Multispectral FX imager. The intravenous injected adenovirus usually harbored in the liver (>90%). In the non-tumor bearing mice that were injected with the original unmodified Ad-INSM1p-*Luc2*, it was observed that non-specific luciferase expression occurred and was focused primarily in the liver area. In contrast, both the first and second generation modified Ad-INSM1p-*Luc2* did not exhibit non-specific luciferase expression after luciferin administration. To further demonstrate that the modified Ad-INSM1p-*Luc2* virus maintains NE tumor specificity, an Ad-INSM1p-*Luc2* pre-infected H1155 NE tumor was established in nude mice and showed readily tumor imaging by luciferase (Fig. 1I). These results determined that the modified INSM1-promoter (both first and second generation) is essential in blocking the effects of adenoviral regulatory elements to retain tumor specificity *in vivo*.

Ad-INSM1p-Met displays INSM1 specificity in vitro. We constructed an adenoviral vector to express secreted *Metridia* luciferase specifically driven by the modified INSM1-promoter for the detection of NE tumors. Secreted *Metridia* was measured *in vitro* by co-infecting tumor cells with Ad-INSM1p-*Met* vector (Ad-HS4ins-INSM1p-2xNRSE-*Metridia*) and Ad-SV40-*Luc2*. The addition of Ad-SV40-*Luc2* virus was used to normalize the infection efficiency using the ratio between extracellular and intracellular luciferase activity. Both INSM1-negative and INSM1-positive tumor cell lines including lung carcinoma, neuroblastoma, medulloblastoma, pheochromocytoma, and insulinoma were infected with Ad-INSM1p-*Met*/Ad-SV40-*Luc2* for 48 h (Fig. 2). The secreted luciferase activity in the media was readily detected in all of

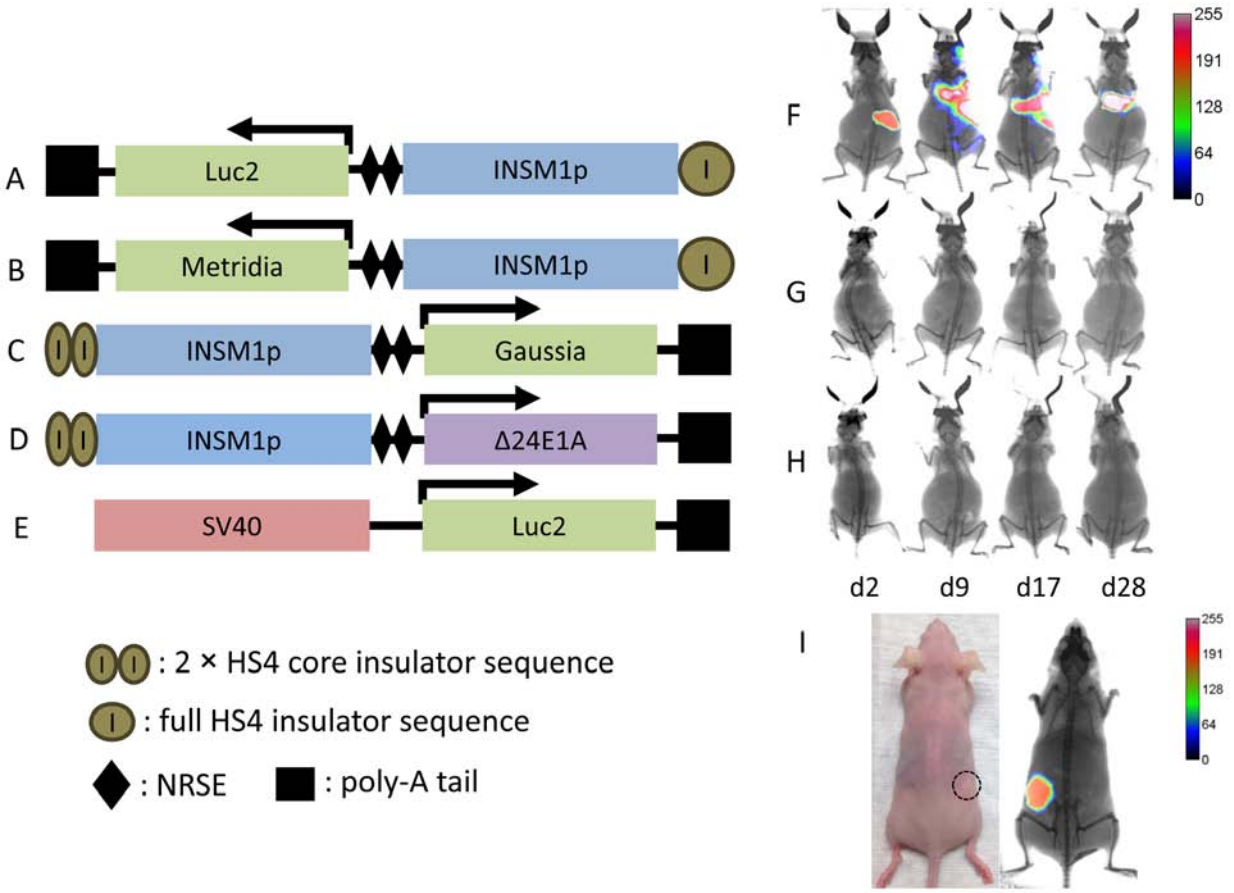

Figure 1. Adenoviral expression vectors. Adenoviral expression vectors were constructed using the SV40 and the modified INSM1 promoter. (A) The INSM1-promoter driven *firefly* luciferase 2 vector was constructed using the first generation modified INSM1-promoter with a 1.2-kb HS4 insulator sequence and 2 copies of the NRSE regulator sequence (22). (B) The *Metridia* luciferase expression vector used the same first generation modified INSM1-promoter for the expression of *Metridia* luciferase. (C) The second generation INSM1-promoter was constructed using two tandem repeats of a 250-bp HS4Core insulator sequence in place of the full insulator sequence. This modified promoter was used to drive expression of *Gaussia* luciferase and (D) *Δ24E1A*. (E) For positive control, the SV40 promoter was used to regulate the expression of *firefly* luciferase 2. For *in vivo* luciferase imaging, Nu/Nu mice aged 8-10 weeks received intravenous tail vein injection of either the unmodified or modified Ad-INSM1p-*Luc2*. (F) To test the specificity, unmodified INSM1-promoter driven Ad-INSM1p-*Luc2* ($1x10^9$) was injected intravenously into non-tumor bearing Nu/Nu mice and the luciferase signal was measured from days 2 to 28 post injection. (G) The first generation and (H) second generation modified INSM1-promoter driven Ad-INSM1p-*Luc2* ($1x10^9$) do not show luciferase activity as INSM1 promoter retains its specificity. (I) For the NE tumor imaging, the modified Ad-INSM1p-*Luc2* virus was pre-infected with H1155 NE lung tumor cells (50 MOI) and injected subcutaneously into the right hind flank of nude mice (n=3). After one week, the tumor growth was evidenced and imaged to show the modified INSM1 promoter specificity. Mouse tumor was faced down for luciferase imaging.

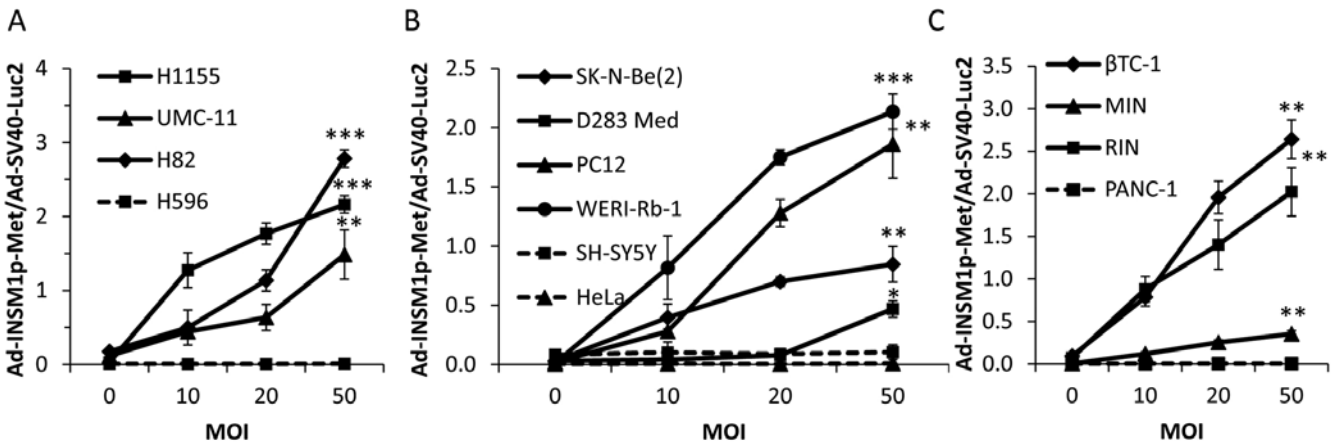

Figure 2. Ad-INSM1p-*Met* vector expressed *Metridia* luciferase specifically in INSM1-positive cell lines. An increasing Ad-INSM1p-*Met* concentration (0-50 MOI) and a constant Ad-SV40-*Luc2* concentration (5 MOI) was used to infect INSM1-positive (solid lines) and -negative (dot lines) cell lines in culture. (A) NE lung cancer H1155, UMC-11, H82, and lung adenosquamous carcinoma H596 cells; (B) Neuroblastoma SK-N-Be(2) and SH-SY5Y, retinoblastoma WERI-Rb-1, pheochromocytoma PC-12, medulloblastoma D283 Med, and cervical adenocarcinoma HeLa cells; (C) Insulinoma βTC-1, MIN, RIN, and pancreatic epithelioid carcinoma PANC-1 cells were used. Values are expressed as ratios between extracellular and intracellular luciferase activity. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (n=3).

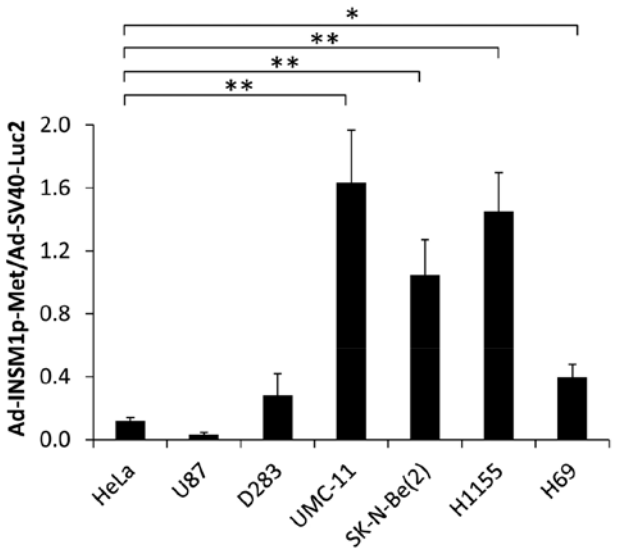

Figure 3. *Metridia* luciferase secretion in xenograft human tumor culture. Ad-INSM1p-*Met* vector specifically expressed *Metridia* luciferase in *ex vivo* NE tumor cells. Cells harvested from established xenograft human tumors were infected with Ad-INSM1p-*Met* (100 MOI) and AdSV40-*Luc2* (100 MOI). Data points are displayed as ratios between extracellular and intracellular luciferase luminescence. *p<0.05, **p<0.01, ***p<0.001 (n=4).

the INSM1-positive cell lines. In particular, the INSM1-positive cell lines H82, βTC-1, and WERI-Rb-1 exhibited the highest Ad-INSM1-*Met*/Ad-SV40-*Luc2* luminescence ratios. At the highest MOI (50:1), secreted luciferase activity reached >2-fold that of the intracellular luciferase activity (Fig. 2). In contrast, the INSM1-negative tumor cell lines showed no secreted *Metridia* luciferase relative to intracellular luciferase.

In order to assess the efficacy of INSM1-promoter driven *Metridia* luciferase adenoviral vector in xenograft human tumors, human tumor cultured cells derived from previously established xenograft tumor were collected and grown in culture. These *ex vivo* tumor cells were co-infected with Ad-INSM1p-*Met* and Ad-SV40-*Luc2* to determine the ratio between extracellular and intracellular luciferase activity. After incubation for 3 days, it was determined that INSM1-positive cells [UMC-11, SK-N-Be(2), H1155, H69, except D283] infected by Ad-INSM1p-*Met* expressed extracellular *Metridia* luciferase that produced signals as high as 1.6 times the intracellular *firefly* luciferase (Fig. 3). In contrast, INSM1-negative cells (HeLa and U87) produced low levels of extracellular *Metridia* luciferase that did not exceed 0.11 times the activity of intracellular *firefly* luciferase.

INSM1 promoter-driven Gaussia luciferase retains specificity. In preparation for further *in vivo* assays, we switched from *Metridia* luciferase to a *Gaussia* luciferase expression vector due to the increased stability of *Gaussia* luciferase *in vivo*. As recent studies have shown, *Gaussia* luciferase signals were detectable after tail-vein injection in mice, due to its increased temperature stability compared to *Metridia* luciferase (19). To determine the specificity of our newly constructed *Gaussia* luciferase construct (Ad-INSM1p-*Gau*), we conducted an *in vitro* luciferase assay to evaluate whether our Ad-INSM1p-*Gau* vector (Ad-2xHS4Core-INSM1p-2xNRSE-*Gaussia*) could specifically express *Gaussia* luciferase in INSM1-positive cell lines in a similar manner as our Ad-INSMp-*Met* vector. After co-infection with Ad-INSM1p-*Gau*/Ad-SV40-*Luc2*, we were able to see significant secreted luminescent activity in the media of all INSM1-positive cell lines as compared to INSM1-negative control cell lines (Fig. 4). In particular, the INSM1-positive cell lines βTC-1, RIN, H82, and H1155 exhibited the highest Ad-INSM1-*Gau*/Ad-SV40-*Luc2* ratios. Secreted *Gaussia* luciferase was not detected in any INSM1-negative cell lines, indicating INSM1 promoter retains specificity *in vitro*.

Conditional replicating vector (Ad-INSM1p-Δ24E1A) enhances Gaussia luciferase secretion and sensitivity over time in an in vivo mouse xenograft tumor model. After establishing the specificity of the Ad-INSM1p-*Gau* vector, we attempted to determine whether infecting cells with

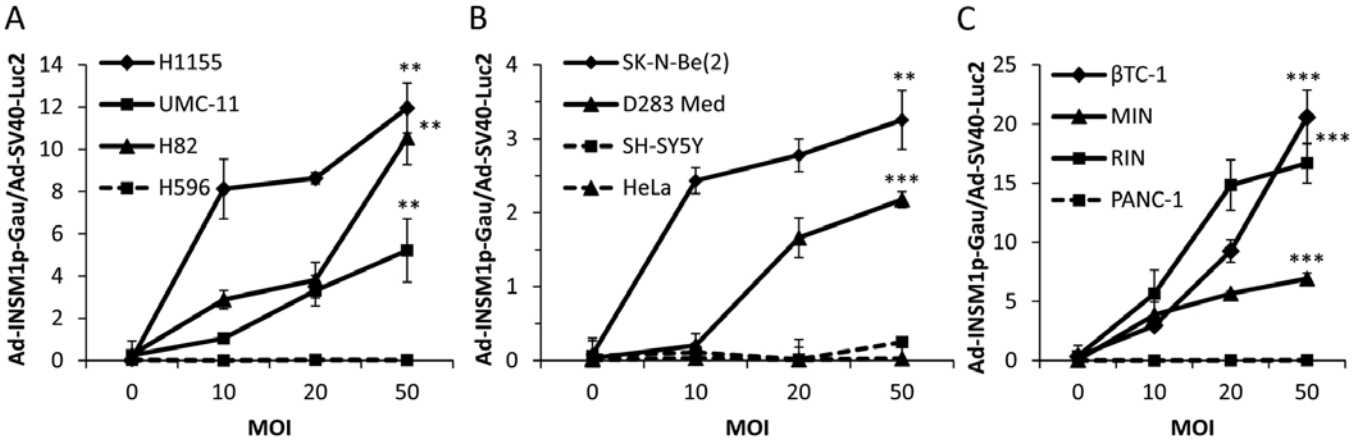

Figure 4. Ad-INSM1p-*Gau* vector expressed *Gaussia* luciferase specifically in INSM1-positive cell lines. An increasing Ad-INSM1p-*Gau* concentration (0-50 MOI) and a constant AdSV40-*Luc2* concentration (5 MOI) was used to infect INSM1-positive (solid lines) and -negative (dot lines) cell lines in culture. (A) NE lung cancer H1155, UMC-11, H82, and lung adenosquamous carcinoma H596 cells; (B) Neuroblastoma SK-N-Be(2) and SH-SY5Y, medulloblastoma D283Med, and cervical adenocarcinoma HeLa cells; (C) Insulinoma βTC-1, MIN, RIN, and pancreatic epithelioid carcinoma PANC-1 cells were used. Values are expressed as ratios between extracellular and intracellular luciferase activity. *p<0.05, **p<0.01, ***p<0.001 (n=3).

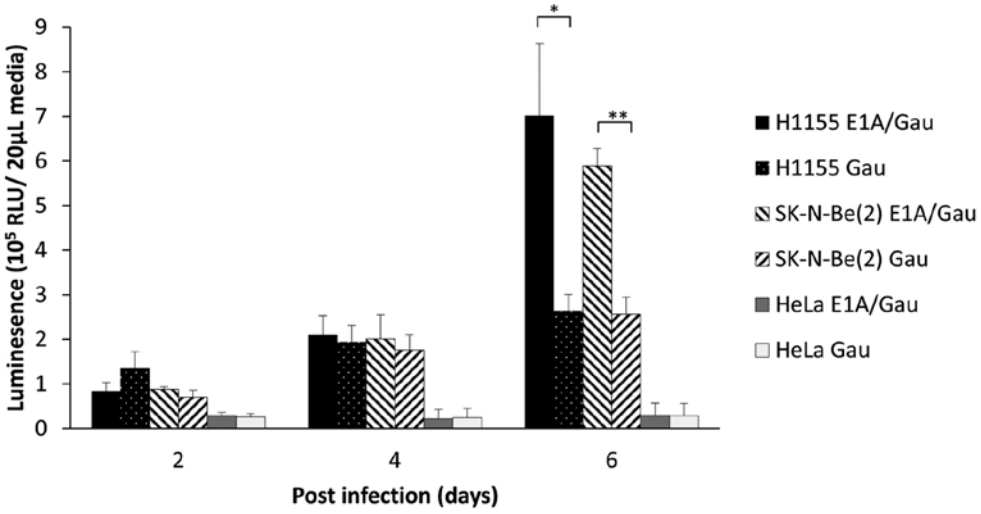

Figure 5. Oncolytic virus in combination with Ad-INSM1p-*Gau* increases sensitivity of *Gaussia* luciferase detection. The combination of Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* was able to increase the sensitivity of luciferase detection after 6 days post-infection. To test the effects of Ad-INSM1p-*Gau* in combination with the conditionally replicating adenovirus Ad-INSM1p-*Δ24E1A*, cells were infected with combination of 10 MOI Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* (a total of 20 MOI). Media were collected 2, 4, and 6 days after infection and showed the highest luciferase activity at day 6. $^{*}p<0.05$, $^{**}p<0.01$ (n=3).

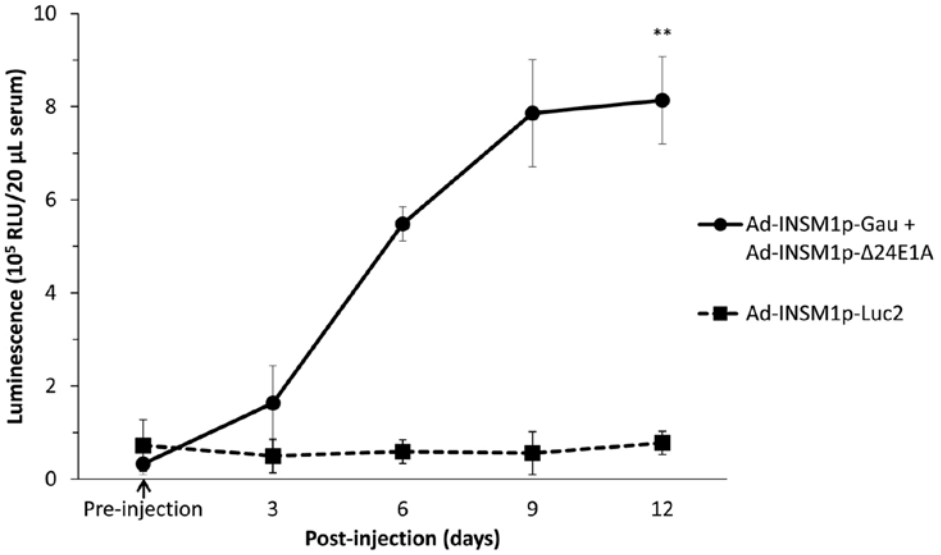

Figure 6. *In vivo Gaussia* secretion assay in a mouse xenograft tumor model. The combination of Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* was able to express detectable *Gaussia* luciferase in circulation 12 days after infection *in vivo*. A mixture of Ad-INSM1p-*Gau* in combination with the Ad-INSM1p-*Δ24E1A* conditionally replicating adenovirus ($1x10^9$ ifu total) was injected into established xenograft tumors (H1155 NE lung cancer cells). Serum was collected from each animal (n=3) 3-12 days post-infection. $^{**}p<0.01$ (n=3).

Ad-INSMp-*Δ24E1A* in combination with Ad-INSM1p-*Gau* could increase the secreted luciferase signal. We hypothesized that if an INSM1-positive cell is infected concurrently with both viruses, *Δ24E1A* expression from Ad-INSM1p-*Δ24E1A* can be utilized by both viruses to facilitate replication. The replication of Ad-INSM1p-*Gau* should lead to viral amplification and increased *Gaussia* luciferase secretion over time. Therefore, we co-infected H1155 NE lung carcinoma cells or SK-N-Be(2) neuroblastoma cells with Ad-INSM1p-*Gau* alone (20 MOI) or in combination with Ad-INSM1p-*Δ24E1A* at a concentration of 10 MOI each (Fig. 5). Six days after infection, the combination viruses displayed a 2-fold increase in secreted luminescent activity as compared to infection with Ad-INSM1p-*Gau* alone. This result suggested that our combination viruses could indeed amplify inside an INSM-positive cell *in vitro*.

We further analyzed whether the combination of Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* viruses could secrete detectable amount of Gaussia luciferase into the circulation from a tumor-bearing animal for an extended period of time. In this experiment, subcutaneous H1155 tumors (~0.1 cm^3) were first established on the right flank of Nu/Nu mice. These tumor-bearing mice (n=3) were then injected intra-tumorally with either Ad-INSM1p-*Luc2* or the

Ad-INSM1p-*Gau* and Ad-INSM1p-*Δ24E1A* virus combination at a total concentration of $1x10^9$ ifu (Fig. 6). After infection with the virus combination for 6 days, detectable luciferase signal was observed in the serum ($p<0.01$). The signal increased in intensity by day 9 and lasted up to 12 days, the humane endpoint for the tumor bearing animals. The Ad-INSM1p-*Luc2* infected tumor released no *Gaussia* luciferase into the circulation and was used as the control.

Discussion

Although the original INSM1-promoter possesses NE-tumor specificity, it was discovered that the promoter loses its specificity when used in an adenoviral setting. In a recent study, Akerstrom *et al* demonstrated that an INSM1-promoter driven adenoviral reporter construct displayed non-specific expression after tail vein injection in an *in vivo* mouse model (22). It was hypothesized that this loss of specificity was due to the presence of overpowering viral enhancers that were otherwise not present in normal cells. To override these adenoviral regulatory elements, an insulator sequence derived from the HS4 chicken β-globin insulator was placed upstream of the INSM1-promoter to block effects from any viral enhancers. In addition, two copies of the neuron-restrictive silencer element (NRSE), a regulatory element with dual functions to silence the INSM1 promoter in non-neuronal cells while enhancing it in neuronal cells, were placed downstream of the promoter. Once these elements were added, the modified INSM1-promoter was able to retain its high specificity in an adenoviral vector (22). To further improve upon this original design, the present study replaced the 1.2-kb full insulator sequence with two copies of the HS4 core insulator (250 bp x 2) to create the second generation modified INSM1 promoter. Although the 1.2-kb full insulator sequence has been well characterized functionally, the 250-bp core insulator was observed to exhibit the same protective activity as the full sequence (23). The main benefit of switching from a full insulator sequence to the core sequence is that utilization of two copies of the 250-bp core would free ~700 bp of space for the assembly of larger transgenic sequences in the viral vector. Essentially, this more compact form of the modified INSM1-promoter displays the same NE tumor specificity with the additional advantage of allowing more flexible cloning strategies.

Retaining the specificity of the INSM1-promoter in an adenoviral vector has allowed us to construct a *Gaussia* luciferase reporter vector that can detect the presence of NE tumor *in vivo*. When paired with a conditionally replicating oncolytic virus, the virus combination allowed for continuous expression of *Gaussia* luciferase for the duration of the tumor's progression. These results could have a significant impact on monitoring tumor progression during the treatment of patients. Given that the viruses can selectively replicate in NE tumor cells, *Gaussia* luciferase expression should persist and intensify as the tumor increases in size. Conversely, if treatment of the tumor is successful, luciferase expression in the patient's blood should decrease as tumor size is reduced. Our study is a proof in principle that the *Gaussia* vector can be used in combination with a treatment protocol to monitor a patient's treatment outcome. An alternative use for this virus during the treatment of a NE tumor would be to discern whether a tumor is removed completely after surgical resection. By injecting the virus combination into the resection site during the surgical procedure, clinicians would be able to monitor the presence of INSM1-positive NE tumor cells based on a *Gaussia* luciferase readings from the patient's blood. Continuous monitoring of expression levels would allow for a better prognosis in these patients post-procedure by alerting clinicians to an incomplete resection.

Using the Ad-INSM1p-*Gau* vector in combination with the Ad-INSM1p-*Δ24E1A* was discovered to be more advantageous as compared to using Ad-INSM1p-*Gau* alone. In NE tumor cells infected by the virus combination, *Gaussia* luciferase expression was significantly higher than that of the Ad-INSM1p-*Gau* virus (20 MOI) alone after 6 days post-infection, even though the number of infectious units of Ad-INSM1p-*Gau* (10 MOI) was lower at the start for the combination. This indicates that the addition of *Δ24E1A* expression in cells infected by our *Gaussia* virus allowed for conditional replication of the reporter vector. This replication has the potential to significantly increase the copy number of the virus over several days, leading to an increase in sensitivity of *Gaussia* luciferase detection. Therefore, the most efficient method of increasing the sensitivity of infection seems to involve utilization of conditionally replicating viruses, as opposed to simply increasing the infectious units during administration of the virus.

Taken together, the Ad-INSM1p-*Gau* virus has the potential to be an easy-to-use and highly sensitive tool for the detection of NE tumors in the clinical setting. While a viral construct cannot be used as a diagnostic tool for the general population, it can be an alternative approach to track the tumor progression in patients with existing NE cancers. Additionally, it could also be used diagnostically in populations where a NE tumor is suspected. In these cases, the virus combination could act as both a diagnostic tool and as a way to monitor tumor progression.

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