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TITLE:

Repression of Multiple Myeloma Cell Growth *In Vivo* by Single-wall Carbon Nanotube (SWCNT)-delivered MALAT1 Antisense Oligos

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SUMMARY:

This manuscript describes the synthesis of a single-wall carbon nanotube (SWCNT)-conjugated MALAT1 antisense gapmer DNA oligonucleotide (SWCNT-anti-MALAT1), which demonstrates the reliable delivery of the SWCNT and the potent therapeutic effect of anti-MALAT1 *in vitro* and *in vivo*. Methods used for synthesis, modification, conjugation, and injection of SWCNT-anti-MALAT1 are described.

ABSTRACT:

The single-wall carbon nanotube (SWCNT) is a new type of nanoparticle, which has been used to deliver multiple kinds of drugs into cells, such as proteins, oligonucleotides, and synthetic small-molecule drugs. The SWCNT has customizable dimensions, a large superficial area, and can flexibly bind with drugs through different modifications on its surface; therefore, it is an ideal system to transport drugs into cells. Long noncoding RNAs (lncRNAs) are a cluster of noncoding RNA longer than 200 nt, which cannot be translated to protein but play an important role in biological and pathophysiological processes. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved lncRNA. It was demonstrated that higher MALAT1 levels are related to the poor prognosis of various cancers, including multiple myeloma (MM). We have revealed that MALAT1 regulates DNA repair and cell death in MM; thus, MALAT1 can be considered as a therapeutic target for MM. However, the efficient delivery of the antisense oligo to inhibit/knockdown MALAT1 *in vivo* is still a problem. In this study, we modify the

SWCNT with PEG-2000 and conjugate an anti-MALAT1 oligo to it, test the delivery of this compound *in vitro*, inject it intravenously into a disseminated MM mouse model, and observe a significant inhibition of MM progression, which indicates that SWCNT is an ideal delivery shuttle for anti-MALAT1 gapmer DNA.

INTRODUCTION:

The SWCNT is a novel nanomaterial that can deliver various types of drugs, such as proteins, small molecules, and nucleic acids, stably and efficiently with ideal tolerability and minimum toxicity *in vitro*[1] and *in vivo*[2]. A functionalized SWCNT has great biocompatibility and water solubility, can be used as a shuttle for smaller molecules, and can carry them to penetrate the cell membrane[3-5].

lncRNAs are a cluster of RNA (> 200 nt) that are transcribed from the genome to mRNA but cannot be translated to proteins. Increasing evidence has shown that lncRNAs participate in the regulation of gene expression[6] and are involved in the initiation and progression of most types of cancer, including MM[7-9]. MALAT1 is a nuclear-enriched noncoding transcript 2 (NEAT2) and a highly conserved lncRNA[10]. MALAT1 is initially recognized in metastatic non-small-cell lung cancer (NSCLC)[11], but has been overexpressed in numerous tumors[5,12-13]; it is one of the most highly expressed lncRNAs and is correlated with a poor prognosis in MM[8,14]. The expression level of MALAT1 is significantly higher in fatal course extramedullary MM patients compared to those only diagnosed as MM[15].

In a previous study, we have confirmed that anti-MALAT1 oligos robustly lead to DNA damage and apoptosis in MM[16] by using gapmer DNA antisense oligonucleotides targeting MALAT1 (anti-MALAT1) in MM cells. The gapmer DNA is composed of antisense DNA and linked by 2'-OMe-RNAs, which could prompt MALAT1 cleavage by RNase H activity once bound[17]. The *in vivo* delivery efficiency of antisense oligos still limits its clinical usage.

To test the delivery effect of SWCNT for anti-MALAT1 gapmer oligos, the anti-MALAT1 gapmer DNA is conjugated to DSPE-PEG2000-amine functionalized SWCNT. The SWCNT-anti-MALAT1 is then injected intravenously into an MM disseminated mouse model; a striking inhibition is observed after four treatments.

PROTOCOL:

All experiments involving animals were pre-approved by the Cleveland Clinic IACUC (Institutional Animal Care and Use Committee).

1. Synthesis of Functionalized SWCNTs

1.1. Mix 1 mg of SWCNTs, 5 mg of DSPE-PEG2000-Amine, and 5 mL of sterilized nuclease-free water in a glass scintillation vial (20 mL). Shake it well to dissolve all reagents completely.

1.2. Sonicate the vial in a water bath sonicator at a power level of 40 W for 1 h at room temperature (RT, 20 min x 3, change the water every 20 min to avoid overheating). Then,

centrifuge it at 24,000 x *g* for 6 h and collect the supernatant solution. This functional SWCNT solution can be stored at 4 °C for 2 months.

1.3. Add 1 mL of SWCNT solution from step 1.2 to a 100-kDa MWCO centrifugal filter device, followed by 4 mL of sterilized nuclease-free water. Centrifuge for 10 min at 4,000 x *g* at RT to remove extra DSPE-PEG2000-amine. Repeat the addition of 4 mL of water and the centrifugation 5x. More than 0.5 mL of leftover SWCNT solution will be left in the filter after each centrifugation.

1.4. Measure the concentration of the SWCNT solution leftover in the filter using a UV/VIS spectrometer at 808 nm after the final wash. The final concentration is typically about 50 mg/L (calculated according to the method developed by Kam *et al.*[18]).

Note: Ensure that DSPE-PEG-functionalized SWCNTs are water-soluble after step 1.4 and are stable in different biological solutions without visible aggregation after a few weeks.

2. Conjugation of Anti-MALAT1-1/2 Gapmer DNA Flanked by 2'-O Modified RNAs to Functionalized SWCNT

2.1. Add 0.5 mg of Sulfo-LC-SPDP into 450 µL of DSPE-PEG2000-amine functionalized SWCNT. Add 50 µL of 10x PBS and then incubate for 2 h at RT.

2.2. To remove extra Sulfo-LC-SPDP, add the reaction from step 2.1 to a 100-kDa MWCO centrifugal filter device, followed by 4 mL of nuclease-free water. Centrifuge for 10 min at 4,000 x *g* at RT. Repeat the addition of 4 mL of water and the centrifugation 5x.

2.3. Add 200 nM thiol-modified anti-MALAT1-Cy3 gapmer DNA into 1.5 mL of commercial DTT solution and incubate for 90 min at RT. Purify the product with a NAP-5 column, following the manufacturer's protocol. Elute the anti-MALAT1-Cy3 with 500 µL of sterilized nuclease-free 1x PBS.

Note: The functionalized SWCNT can be stored with added DTT, which might cleave disulfide bonds formed during the storage of the thiolated anti-MALAT1 gapmer DNA. The added DTT can be removed by a NAP-5 column before conjugation with SWCNT.

2.4. Collect the sulfo-LC-SPDP-treated DSPE-PEG2000-amine SWCNT solution left in the filter and dilute it with 500 µL of anti-MALAT1-Cy3 solution. Then, incubate it at 4 °C for 24 h. The conjugated SWCNT-anti-MALAT1 can be stored at 4 °C for 3 weeks.

3. Tail Vein Injection of SWCNT-Anti-MALAT1

3.1. Generate an MM disseminated mouse model.

3.1.1. To achieve the best results of comparison, randomly arrange 14 NOD.CB17-Prkdcscid/J mice (8 weeks old) into trial or control groups (seven in each group) with the same male/female ratio.

3.1.2. Clean the operation bench and sterilize it with 70% alcohol.

3.1.3. Use a heating lamp to warm the mouse to help the tail vein to appear. Restrain the mouse properly with a tail injection restrainer.

3.1.4. Inject 5 x 10^6 H929-Luc-mCherry cells in 50 µL of PBS through the tail vein without anesthesia. Press the injection site with an alcohol swab for 30 s. Mark the injected mouse and return it to a clean cage.

3.2. Start the treatment on day 7 after the H929 cell injection.

3.2.1. Inject 50 µL of PBS containing SWCNT-anti-MALAT1 into the tail vein of the mouse. Use PBS containing SWCNT-control as a control.

3.2.2. Press the injection site with an alcohol swab for 30 s.

3.2.3. Observe the local bleeding on the tail and the general behavior of the mouse for 1 min, and then return it to a clean cage. Observe all injected mice again before returning the cage to the rack, to make sure that the injections are tolerated well.

3.3. Repeat the injection every 7 d until the termination of the experiment.

3.3.1. Terminate the experiment when the general health of the mouse degrades: when not eating or drinking, the appearance of pale paws, any subcutaneous bleeding, a shortness of breath, decreased activity, a paralysis of the lower limbs, and/or a touchable mass in the abdomen is observed.

4. Evaluation of the Disease Progression

4.1. Assess the general status of the mice every day after the H929-Luc-mCherry cell injection. Pay particular attention if the mice develop paralyzes of lower limbs.

4.2. Evaluate the tumor growth using an *in vivo* imaging system 1x a week, before the SWCNT-anti-MALAT1 injection.

4.2.1. Prepare fresh 15 mg/mL D-Luciferin with 1x PBS.

4.2.2. Anesthetize the mice with an isoflurane vaporizer (3% - 5% for induction and 1% - 3% for maintenance, depending on the status of the mice).

4.2.3. Inject 150 mg/kg D-Luciferin in each mouse intraperitoneally, 5 min before imaging; then, image the mice in a prone position with an spectrum imaging system.

4.2.4. Collect sequential images of the mice every 2 min, until luminescence saturation is reached. Adjust the interval time according to the D-Luciferin uptake/signal.

4.3. Use CO_2 to sacrifice the mice once the termination of the experiment is reached.

4.4. Since this is an MM disseminated model, process the mice as follows.

4.4.1. Weigh the mouse before dissection.

4.4.2. Collect peripheral blood from the heart for a complete blood count (CBC) assay performed by a blood cell counter machine. The counts of white blood cells and red blood cells, as well as the ratio of lymphocytes, are the primary indexes.

4.4.3. Isolate the spleen and weigh it. Calculate the ratio of spleen/body weight; consider > 0.5% as spleen enlargement.

4.4.4. Collect the tissues of bone marrow, spleen, lymph node, kidney, and the tissue with visible metastasis.

4.4.5. Collect the spine if the mouse developed a paralysis of the lower limbs.

4.4.6. Extract RNA and protein from the bone marrow samples.

4.4.7. Fix all remaining tissues in formalin.

4.4.8. For decalcification, immerse the bone samples in 0.25 M EDTA solution (pH 8.0) for 5 d after 24 h of fixation.

4.4.9. Immerse all samples in 75% alcohol for long-term storage.

4.5. Compare the signal strength of luciferase at the same time points in two groups. From both groups, record the sacrifice dates of each mouse and calculate the survival curves of the two groups with software.

Note: All procedures are summarized in **Figure 1**.

REPRESENTATIVE RESULTS:

To demonstrate the inhibition effect of anti-MALAT1 gapmer DNA in MM, we knocked down the expression of MALAT1 and used it in H929 and MM.1S cells. Forty-eight hours later, cells were collected for the analysis of knock-down efficiency and the apoptosis status in cells transfected with anti-MALAT1 gapmer or control DNA. qRT-PCR results showed that anti-MALAT1 gapmer

DNA knocked down the MALAT1 expression in H929 and MM.1S cells efficiently (**Figure 2A**). The status of apoptosis was determined by flow cytometry, which revealed down-regulated MALAT1 induced apoptosis significantly (**Figure 2B**).

These results indicate that anti-MALAT1 oligos inhibit MM effectively *in vitro*. However, an efficient delivery method/shuttle for anti-MALAT1 oligos *in vivo* needed to be developed, which is also the obstacle of antisense oligos in clinical application; hence our interest in SWCNT. SWCNT is a novel nanomaterial for drug delivery, which can develop hydrophilicity and dissolubility after appropriate modifications; therefore, it can deliver cargos in different forms, such as oligonucleotide drugs. To validate the delivery efficiency of SWCNT *in vivo*, we first functionalized the SWCNT with DSPE-PEG2000-amines, which endowed hydrophilicity and binding specificity to the SWCNT[19]. Then, this functional SWCNT was treated with sulfo-LC-SPDP to create free sulfhydryl groups, which will be used to connect with the oligo. To conjugate the anti-MALAT1 oligo and the sulfo-LC-SPDP-treated SWCNT, the 5' ends of the anti-MALAT1 oligo has been modified with thiol groups (S-S); to visibly track the delivery, the 3' ends of the anti-MALAT1 oligo was modified with cyanine 3 (Cy3). After all the synthesis steps, SWCNT-anti-MALAT1-Cy3 was obtained (**Figure 1**)[12]. Then, it was added to the culture medium of H929-GFP and MM.1S-GFP cells to validate the delivery efficiency.

As shown in **Figure 2C**, SWCNT-anti-MALAT1-Cy3 was efficiently delivered into the nucleus of MM cells and significantly suppressed the endogenous MALAT1 level in both H929 and MM.1S cells (**Figure 2D**). To examine the toxicity and safety of SWCNT in normal cells, SWCNT-anti-MALAT1-Cy3 was added in the medium of BMEC-1 cells at different concentrations; Lipofectamine was the treatment control; plain medium was used as the essential control (**Figure 2E**). Then, cell viability was detected using a cell viability assay kit on day 1, day 2, and day 3. No significant difference in cell viability inhibition was found between SWCNT-anti-MALAT1-Cy3 and the treatment control at different dosages and time points. There was no difference compared to plain medium (NC) either, which indicates that the toxicity of SWCNT-anti-MALAT1-Cy3 is similar to the treatment control, which has a limited and acceptable toxicity to normal cells at high dosage.

Next, a human-MM disseminated mouse model was established to evaluate the treatment effects of SWCNT-anti-MALAT1 *in vivo*. First, each SCID-beige mouse (of 8 weeks old) was injected with 5×10^6 H929-Luc-mCherry cells intravenously (**Figure 3**). A total of 14 mice were injected with H929 cells; among them, seven mice were randomly arranged into an SWCNT-anti-MALAT1-Cy3 treatment group, and another seven were in the control group. Both SWCNT-anti-MALAT1 and SWCNT-control oligos were dissolved in 0.5 mL of 1x PBS and injected through the tail veins 7 days after the tumor cell injection. The SWCNT-anti-MALAT1-Cy3 treatment was repeated every 7 days until the termination of the experiment. To evaluate the tumor burden, the luciferin signal was observed by an *in vivo* imaging system (IVIS) 30 min after the luciferin injection 1x a week before the SWCNT-oligo injection. We found that the luciferin signals of the mice in the SWCNT-anti-MALAT1 treatment group were remarkably lower compared with those of the SWCNT-control treatment group after 21 days of treatment (from day 28). Their health and survival status was checked and recorded every day and summarized in a Kaplan-Meier curve. From these results, we concluded that the SWCNT-anti-MALAT1 treatment *via* intravenous injection can

deliver the anti-MALAT1 oligos to tumor cells effectively. We, then, limited the tumor burden and extended the mice's lifespans ($p = 0.04$) as expected, which is similar to *in vitro* results. We did not find any significant side effects of the treatment in the mice during the whole experiment period, which indicated that the SWCNT-anti-MALAT1 injection is a safe treatment for mice; therefore, SWCNT is a safe and reliable *in vivo* delivery system for anti-MALAT1.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the synthesis, absorption, and *in vivo* dissociation of an SWCNT-anti-MALAT1-Cy3 gapmer oligo. (**A**) SWCNT was functionalized with DSPE-PEG2000-amines and, subsequently, conjugated with anti-MALAT1-Cy3 *via* disulfide linkage mediated by Sulfo-LC-SPDP. (**B**) SWCNT-conjugated anti-MALAT1 was injected into a mouse with H929-Luc-mCherry cells *via* the tail vein. (**C**) SWCNT-conjugated anti-MALAT1 penetrates the phospholipid bilayer membrane through insertion or endocytosis. (**D**) SWCNT-anti-MALAT1 was packaged within early endosomes after absorption by cells; then, the early endosome matured to a late endosome and merged with a lysosome to form an endolysosome. The endolysosome helps release anti-MALAT1 from the SWCNT by breaking the disulfide bond.

Figure 2: SWCNT-anti-MALAT1 was delivered efficiently with minimum toxicity and induced apoptosis in MM cell lines. SWCNT-anti-MALAT1 gapmer oligo or SWCNT-control oligo were transfected by Lipofectamine into H929 and MM.1S cells, respectively. Forty-eight hours later, we collected cells for (**A**) the analysis of MALAT1 expression by qRT-PCR and (**B**) apoptosis analysis by flow cytometry. (**C**) H929-GFP and MM.1S-GFP cells were co-cultured with SWCNT-anti-MALAT1-Cy3 for 48 hours; the delivery efficiency was determined by fluorescence microscope (the scale bars = 100 µm). (**D**) The expression level of MALAT1 was detected by qRT-PCR, which showed that it was successfully knocked down in H929 and MM.1S cells (** $p < 0.01$, *** $p < 0.001$). (**E**) SWCNT and the treatment control were added into a culture medium of BMEC-1 cells at different dosages. The proliferation was measured using a cell viability assay kit. This figure has been modified from Hu *et al.*[16].

A. Figure 3: SWCNT-anti-MALAT1 suppressed myeloma growing in H929-cell-constructed MM disseminated mouse model. (**A**) 5 x 10^6 H929-Luc-mCherry cells were injected intravenously to the tail veins of irradiated SCID-beige mice (seven in each group); 50 µL of an SWCNT-anti-MALAT1 or SWCNT-control solution were injected every 7 days *via* the tail vein from the 7th day after the H929 cell injection. IVIS was used to monitor the tumor growth. (**B**) Hind limb paralysis and tumor burden were used as endpoints, and the survival data were analyzed by a Kaplan-Meier analysis. This figure has been modified from Hu *et al.*[16].

DISCUSSION:

Evidence has shown that lncRNAs take part in the regulation of numerous physiological and pathophysiological procedures in cancers, including MM[7-9]; they have the potential to be targeted for cancer treatment, which can be realized by antisense oligonucleotides[20-22]. The U.S. Food and Drug Administration (FDA) has approved several antisense oligonucleotide drugs, including fomivirsen for cytomegalovirus retinitis[23], mipomersen for homozygous familial

hypercholesterolemia[24], nusinersen for spinal muscular atrophy[25], and eteplirsen for Duchenne muscular dystrophy[26]. In this study, we modified the anti-MALAT1 gapmer DNA with 2'-OMe-RNA and conjugated it with functional SWCNT. The SWCNT carries and delivers anti-MALAT1 oligos as a shuttle, which not only improved the affinity of the oligo-target due to its flexibility and multiple loading but also stabilized the oligos and helped prevent nuclease degradation during delivery[27].

A suitable modification on the surface of SWCNTs can help it get reliable dispersibility in physiologically relevant, aqueous environments and promote their biodistribution[28,29]. We used DSPE-PEG2000-amine to modify the SWCNT, which has been demonstrated to graft on SWCNT and improve the aqueous solubility of it, and furthermore, it showed excellent stability without agglomeration in biological media[30]. Sulfo-LC-SPDP was used as a crosslinker in the experiment, which helped functionalized SWCNT to bind the anti-MALAT1 gapmer DNA through disulfide linkage, and thereafter deliver them into cells. Once the SWCNT penetrated in MM cells, they were taken up by early endosomes (pH ~ 6.0), which then matured to late endosomes (pH ~ 5.0) accompanied by a reduced pH value[31]. The advantage of the PEG link is that the bonding is stable at pH > 6, but up to 75% - 95% of the PEG-linked drug was released within 2 hours once the pH became less than 5.5[32,33,34]. Endosomes carrying SWCNT-anti-MALAT1 merged with lysosomes (pH ~ 4.5) to form an endolysosome, and then the disulfide bonds were catalyzed by lysosomal thiol reductase, which was optimally activated at a low pH[35]. This, subsequently, released free anti-MALAT1 gapmer DNA. According to the experiment results *in vitro* and *in vivo*, the SWCNT delivered SWCNT-anti-MALAT1 effectively and helped it act similarly in function to anti-MALAT1 *in vitro*.

The functionalized SWCNTs pass through phospholipid bilayer *via* two patterns: insertion[36,37] and endocytosis[38,39]. These procedures help the SWCNT deliver cargos into cells without interrupting the stabilization of cell membranes, therefore reduced the toxicity of the SWCNT. We injected 50 µL of SWCNT-anti-MALAT1 (~40 mg/L) in each mouse (~20 g); thus, the final drug concentration was 0.1 mg/kg, which is much lower than the dosage we used in toxicity experiments (2 mg/L and 4 mg/L). As previous results showed, SWCNT has a similar effect as the treatment control (*e.g.*, Lipofectamine) on cell growth and viability at matched dosages. The treatment control is a common reagent for cell transfection and is believed to have a limited and acceptable toxicity to cells; thus, we believe SWCNT only has a minimum toxicity for normal cells.

As a shuttle for antisense oligonucleotides, metabolism is another important consideration for the safety of SWCNT. It has been demonstrated that functionalized SWCNT is mainly excreted through kidneys, without glomerular and tubular toxicity[40]. We did not find kidney dysfunction-related symptoms, such as oliguria, anuria, edema, appearance changes of the kidney, *etc.*, in mice that accepted the SWCNT injection. Thus, SWCNTs have no toxicity effects due to any abnormal accumulation in a mouse with a normal kidney function.

We are the first to conjugate anti-sense oligonucleotides targeting lncRNA with functionalized SWCNT and use it for MM treatment. SWCNT is a type of novel nanomaterial, which has consistent chirality, optional diameter, and length distribution. After functionalization treatment, SWCNTs develop water solubility and biocompatibility and become an ideal biological shuttle to

deliver numerous cargo through the cell membrane, thereby increasing drug distribution and enhancing treatment effect. In addition, SWCNT may stabilize anti-sense oligonucleotide molecules from nuclease digestion[1]. As the results show, the functionalized SWCNT-anti-MALAT1 delivered MALAT1 into MM cells efficiently and inhibited MM growth dramatically in cell lines *in vitro* and in a disseminated mouse model *in vivo*. So far, we did not observe any toxicity due to SWCNT treatment in these experiments. This study demonstrates that SWCNT is a safe and effective delivery vehicle for antisense oligonucleotide drugs and has the potential to be used as a carrier for therapeutic molecules in patients.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure-1

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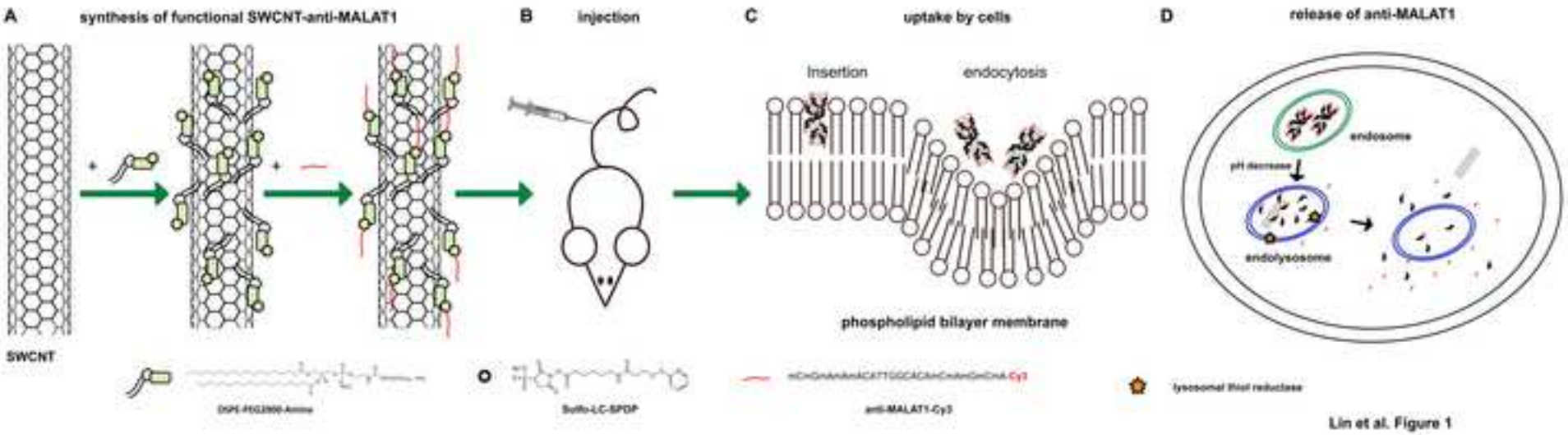

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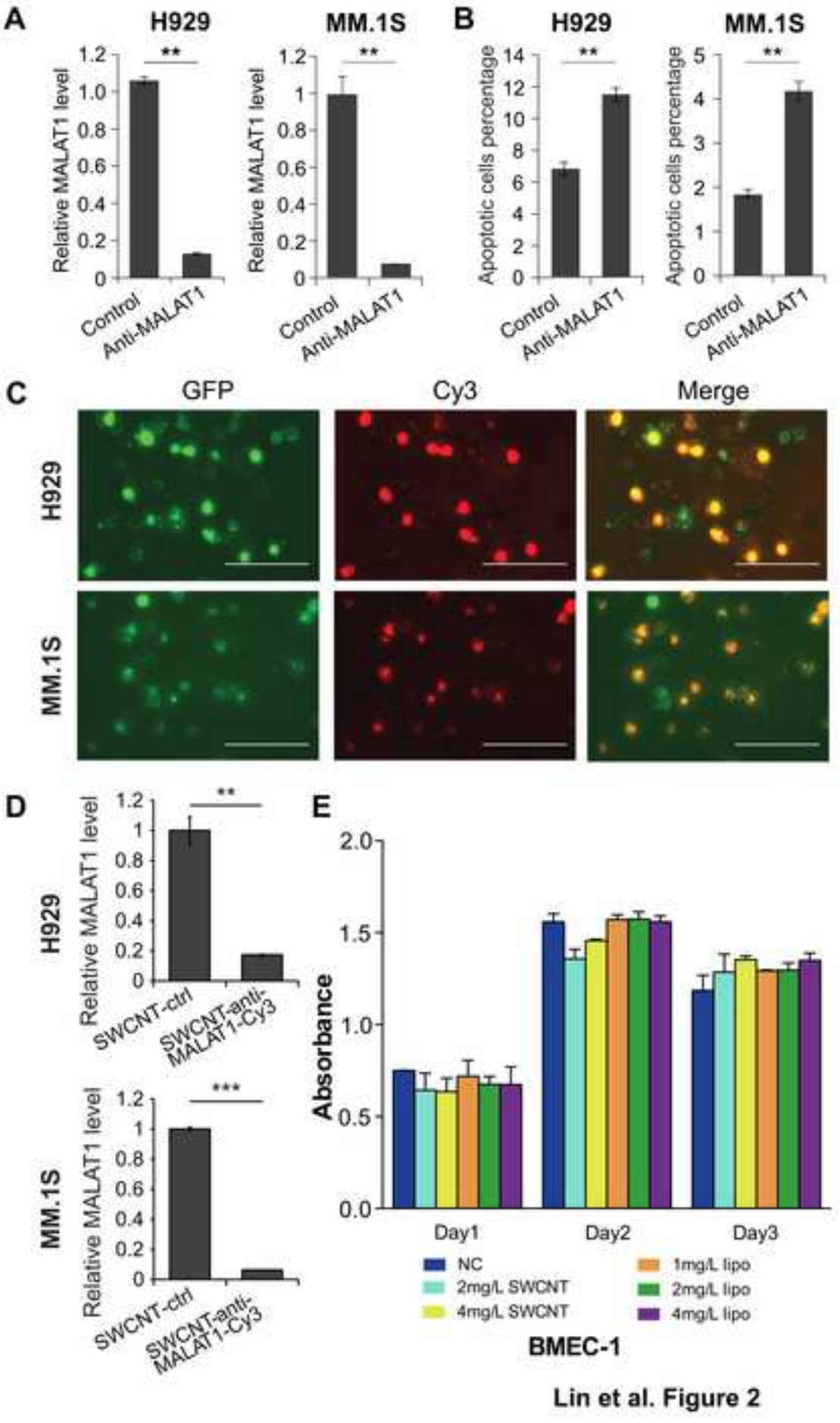

Lin et al. Figure 2

Figure-3

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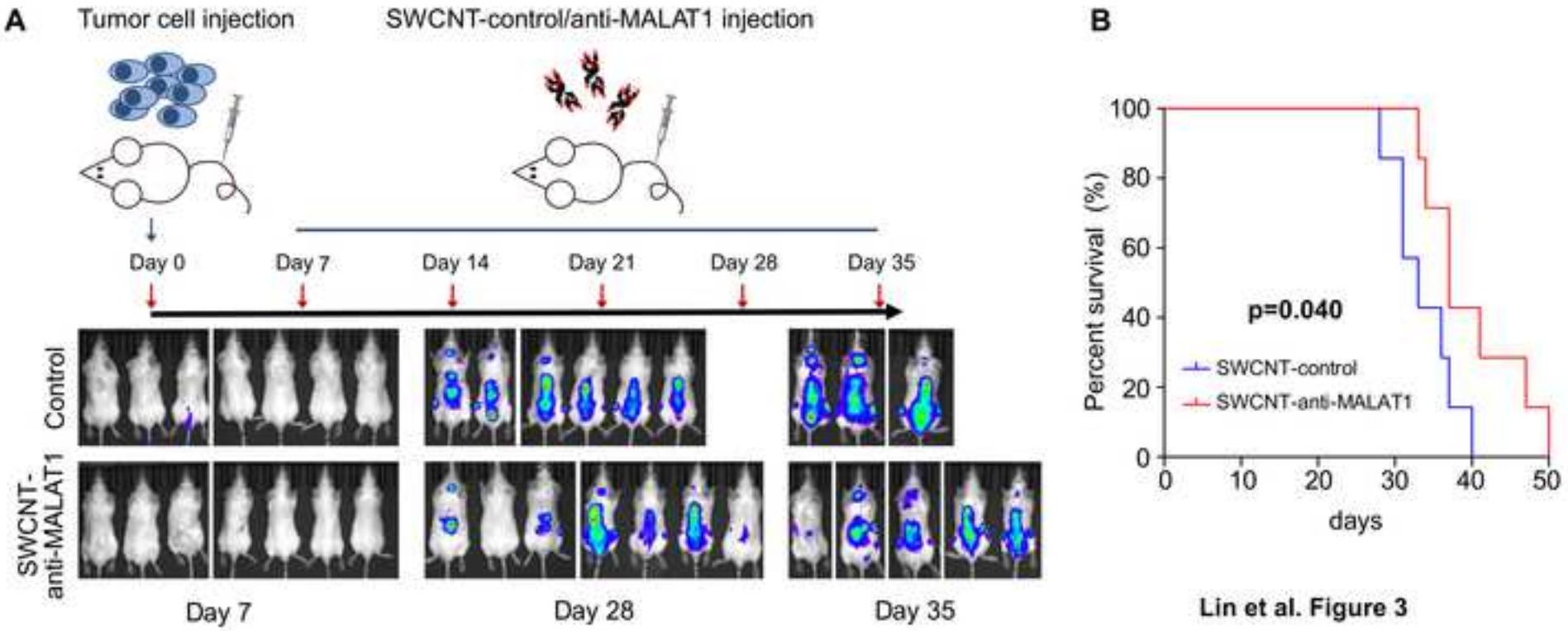

Lin et al. Figure 3

material

Click here to download Table of Materials JoVE_Materials-2018.7.27.xlsx

Name of Material/ Equipment	Company
SWCNTs	Millipore-Sigma
DSPE-PEG2000-Amine	Avanti Polar Lipids
bath sonicator	VWR
4 mL centrifugal filter	Millipore-Sigma
UV/VIS spectrometer	Thermo Fisher Scientific
Sulfo-LC-SPDP	ProteoChem
DTT solution	Millipore-Sigma
NAP-5 column	GE Healthcare
in vivo imaging system	PerkinElmer
NOD.CB17-Prkdcscid/J mice	Charles River lab
Flow cytometer	Becton Dickinso
Lipofectamine	Invitrogen
Fetal bovine serum (FBS)	Invitrogen
RMPI-1640 medium	Invitrogen
MALAT1-QF:	synthesized by IDT Company
MALAT1-QR:	synthesized by IDT Company
GAPDH-QF:	synthesized by IDT Company
GAPDH-QR:	synthesized by IDT Company
Quantitative PCR using SYBR Green PCR master mix	Thermo Fisher Scientific
RevertAid first-stand cDNA synthesis kit	Thermo Fisher Scientific
anti-MALAT1	synthesized by IDT Company
Cell Viability Assay Kit	Promega Corporation

accuSkan GO UV/Vis Microplate Spectrophotometer	Thermo Fisher Scientific
centrifugal filter	Millipore-Sigma
SPSS software	IBM
D-Luciferin	Millipore-Sigma

Catalog Number

704113

880128

97043-992

Z740208-8EA

accuSkan GO UV/Vis Microplate Spectrophotometer

c1118

43815

17-0853-01

250

11668019

10437-028

11875-093

A25780

K1621

G7570

UFC910008
version 24.0
L9504

Comments/Description

extinction coefficient of 0.0465 L/mg/cm at 808 nm

Lipofectamine2000

5’- GTTCTGATCCCGCTGCTATT - 3’

5’- TCCTCAACACTCAGCCTTTATC - 3’

5’- CAAGAGCACAAGAGGAAGAGAG - 3’

5’- CTACATGGCAACTGTGAGGAG - 3’

5’-mC*mG*mA*mA*mA*C*A*T*T*G*G*C*A*C*A*mC*mA*mG*mC*mA-3’
CellTiter-GloLuminescent Cell Viability Assay Kit

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Author(s):	Jianhong Lin, Yi Hu, Jian-Jun Zhao

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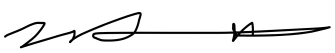
Signature:	
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Dear Dr. Myers,

Thank you for your review our manuscript and give us the opportunity to resubmit our manuscript for your consideration. This resubmission to the JoVE Journal has been revised based on reviewer comments. I am particularly grateful for the critiques and suggestions from the reviewer on the experiments to improve our manuscript. Below, I outline the changes made in the text of our resubmitted version and an underline indicates new or revised text.

Reviewer #1:

Manuscript Summary:

This is an interesting paper, which I think will be highly qualified for JoVE. The author for the first time to conjugate LNA Gapmer antisense oligonucleotides targeting MALAT1 and apply the newly formed ASO in mice treated with tumor cells to investigate the therapeutic function of ASO anti-MALAT1. The results in this paper showed that the conjugated ASO had high efficiency and low toxicity, and could significantly extended the lifespan of tumor cell treated mice.

Major Concerns:

1. Have the author tested that how long does the conjugated oligo could stay in mouse body? Please interpret this point.

There are multiple literatures shown that the ASO conjugated on SWCNT will be released from SWCNT in 2 hours under the action of enzyme in cell lysosomes such as lysosomal thiol reductase (Biomaterials, 34(4):1213-1222). We did not check the retention time of SWCNT-anti-MALAT1 in mice in our study.

2. Concerning the treatment of tumor cells, have the authors tried pretreat the mice with the conjugated ASO anti-MALAT1, which will function as a protection to the mice? Please interpret this point.

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We started to treat the mice in our dissemination mouse model on the day 7 after tumor cell injection, when the tumor cells could be detected by IVIS system. We didn't pretreat the mice with the conjugated ASO anti-MALAT1 because we think it didn't mimic the clinical situation.

3. Is there any comparison between injection of the LNA Gapmer ASO anti-MALAT1 and the conjugated ASO in mice? What is the difference of the amount and efficiency in mice? Please interpret this point.

Since there are studies have demonstrated that, SWCNT conjugated ASO inhibited target gene (mTERT) more intensively than ASO only *in vitro* and *in vivo* (Clinical Cancer Research.12(16):4933-9). We did not compare the treatment efficiency between MALAT1 and SWCNT-anti-MALAT1 directly.

Minor Concerns:
N/A

Reviewer #2:

Manuscript Summary:
The manuscript presents a method for non-viral delivery of antisense oligo targeting the MALAT1 RNA. The study starts with in vitro experiments and then the efficiency of the delivery system is confirmed by in vivo animal studies. The methods are explained step-by-step. The results are discussed properly. The manuscript would help the readers to design similar delivery systems.

Major Concerns:
There are no major concerns.

Minor Concerns:
There are some minor issues that can be explained more. It would be more helpful for the readers, if the following issues were clarified.

1. Line 76: The conversion of absorbance to the concentration could be explained briefly.

The molecular weight of SWCNT is about 170 kDa; the molar extinction coefficient ε is about 7.9×10^6 $M^{-1} \cdot cm^{-1}$ (Proc Natl Acad Sci U S A. 102(33):11600-5). Thus the weight extinction coefficient is about 21.52 $mg \cdot L^{-1} \cdot cm^{-1}$. We measured the absorption at 808 nm wavelength with liquid diameter of 0.5 cm, and the values we got from the UV-visible spectroscopy was about 4.7, thus the final concentration was calculated as 4.7×21.52 $mg \cdot L^{-1} \cdot cm^{-1} \times 0.5$ cm, which was about 50 mg/L.

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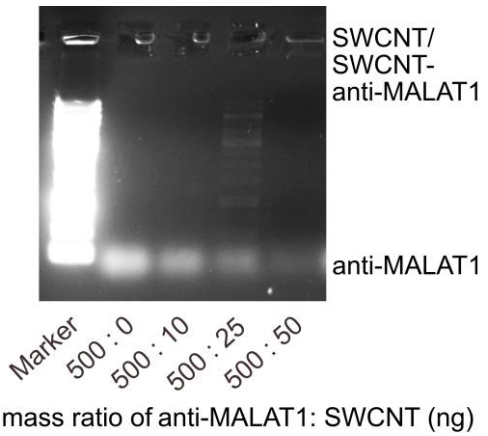
2. Do the authors check the conjugation efficiency of antisense oligo? Do they verify the conjugation?

We checked the optimized mass ratio between anti-MALAT1 and SWCNT conjugation using DNA agarose gel electrophoresis. We found the unconjugated free anti-MALAT1 (lower band) gradually reduced and there is clearly conjugated SWCNT-anti-MALAT1 stay in the gel well (upper band) in 10:1, which is the ratio we used in the experiment. The consumption ratio of NAP-5 column was 40-60 %. The molecular weights of anti-MALAT1 and SWCNT are about 6.5 kDa and 170 kDa and the molar ratio between anti-MALAT1 and SWCNT is about 300:1. Thus, 1 SWCNT molecule binds with 200-400 anti-MALAT1 oligos. This number may be variable because the length and diameter of each SWCNT may be different.



3. Regarding Figüre 1, the cleavable property of the linker could be emphasized in the figüre or in the text. Once inside the cell, the status of the SWCNT conjugate could be simply schematized. For instance, how is the antisense oligo released from the conjugate?

We have added this part in the new figure 1.

Thank you for your consideration!

Sincerely,

Jianjun Zhao, M.D; Ph.D.

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