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Anti-miRNA Oligonucleotide Therapy for Chondrosarcoma

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Abstract

Chondrosarcoma is a highly aggressive primary malignant bone tumor mostly occurring in adults. There are no effective systemic treatments, and patients with this disease have poor survival. MicroRNA-181a (miR-181a) is an oncomiR that is overexpressed in high grade chondrosarcoma, and promotes tumor progression. Regulator of G-protein signaling 16 (RGS16) is a target of miR-181a. Inhibition of RGS16 expression by miR-181a enhances CXCR4 chemokine receptor 4 signaling, which in turn increases MMP1 and VEGF expression, angiogenesis, and metastasis. Here, we report the results of systemic treatment with anti-miRNA oligonucleotides (AMOs) directed against miR-181a utilizing a nanoparticle delivery platform (NP). NP were combined with a molecular beacon or anti-miR-181a oligonucleotides and are shown to transfect chondrosarcoma cells in vitro and in vivo. Intratumoral injection and systemic delivery had similar effects on miR-181a expression in nude mice bearing chondrosarcoma xenografts. Systemic delivery of NP carrying anti-miR-181a also restored RGS16 expression, decreased expression of VEGF and MMP1, MMP activity, and tumor volume by 32% at day 38, and prolonged survival from 23% to 45%. In conclusion, these data support that systemic delivery of AMO shows promise for chondrosarcoma treatment.

Keywords

Nanoparticle; Metastasis; Noncoding RNAs; Bone cancer; microRNA

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Introduction

Chondrosarcoma (CS) remains the only primary bone cancer without an effective systemic treatment. This sarcoma is highly metastatic. Conventional cytotoxic chemotherapy is not effective, and patients typically succumb to pulmonary metastases(1–3). Another approach for systemic treatment is targeted therapeutics, which have yet to be fully developed. A promising targeted approach is manipulation of misexpressed microRNAs(4). MicroRNAs are short, endogenous, non-coding RNAs that negatively regulate gene expression by promoting mRNA degradation or by translational repression through complementarity with sequences in the 3' UTR(5–7). In cancer, microRNAs can function analogous to tumor suppressors or as oncogenes (oncomiRs) when over- or underexpressed, the net effect dependent on the target genes(8;9). In prior work we identified miR-181a as an oncomiR that is upregulated by hypoxia in chondrosarcoma, that in turn upregulates VEGF and MMP expression(10;11). CS cells transfected with a lentivirus expressing anti-miR-181a reversed these downstream effects and decreased lung metastatic burden, however, systemic delivery of microRNAs and anti-miRNA oligonucleotides (AMOs) remain an unsolved problem(4). There are several types of nanoparticles that can be used for drug and AMO delivery including lipid, polymeric, and metallic nanoparticles (12). One challenge to delivery in chondrosarcoma is the negatively charged proteoglycan-rich extracellular matrix that needs to be penetrated to reach the tumor cells. In order to translate our findings into a potential treatment, we performed systemic treatment with AMO directed against miR-181a delivered with a Nanopiece (NP) platform. NP are based on a novel biomimetic molecule: 6-amino fused adenine and thymine, named JBAK (Janus base with Amine or lysine (K) side chain). A JBAK molecule has two components: 1) nucleobases with hydrogen-bond donors and acceptors on two faces respectively, and 2) a hydrophilic side chain containing amine or lysine. We used the lysine side chain in these experiments. With this design, two faces of a Janus Base are complementary to each other and six JBAK molecules form a disc which further self-assembles into a hydrophobic tubular backbone with the positively charged amine or lysine containing side chains remaining on the surface of this tubular structure, thus forming a rosette nanotube: JBNT (Janus Base Nanotube) (13). Through positive-negative charge interaction, and when combined with nucleic acid therapeutics such as siRNA or AMO, and after treatment with ultrasonic energy, they form NP, with a size of 120 × 20 nm(14). NP are nontoxic and can penetrate negatively charged cartilaginous matrix, which is also found in chondrosarcoma(15–18). The pathway and NP delivery of AMO are diagrammed in Figure 1A.

Here, we demonstrate for the first time that 1) NP can deliver nucleotide sequences intracellularly to human tumor cells in vitro and in vivo, and 2) NP carrying AMOs administered systemically inhibit expression of an oncogenic microRNA, restore expression of RGS16, and have a favorable effect on tumor related parameters in a preclinical model.

Materials and Methods

Cell lines and cell culture.

Human chondrosarcoma cell lines CS-1 (a gift from Dr. Francis Hornicek, Harvard Medical School, Boston, MA) and JJ (a gift from Dr. Joel Block, Rush Medical School, Chicago, IL)

cultured with 10% FBS in a humidified incubator (NuAire Inc, Plymouth, MN) under 5% CO₂ and normoxia (ambient oxygen) as previously described (11;18;19). CS-1 was derived from grade III and JJ from grade II human chondrosarcomas respectively; both metastasize in a xenograft mouse model. (11;19;20). The CS-1 cell line was authenticated using short tandem repeat (STR) profiling (ATCC, Manassas, VA) in September 2012, matched the STRS profiling performed by the source laboratory in 2011, and there were no other matches in the ATCC data base. JJ was authenticated using STR profiling on the source cell line in 1999, 2007, and repeated in 2012. There is 94% similarity between the different time points, the cells are human, and there are no matches with any cell lines in the ATCC database. Frozen aliquots of cells were used for this study.

Molecular beacon; oligonucleotides.

The sequence of the molecular beacon for Human GAPDH was 5'-Alexia647-CGACGGAGTCCTTCCACGATACCACGTCG-BHQ3a-3' (Eurofins Genomics, Louisville, KY)(21).

mirVana® miRNA inhibitor specific for human miR-181a and mirVana™ miRNA inhibitor Negative Control #1 were purchased (Thermo Fisher Scientific, Waltham, MA).

Preparation of Nanopieces.

The Janus base nanotubes were synthesized as previously described and dissolved in water to a 1 mg/mL solution(14). For cell culture experiments, NP were generated by mixing 2 µL of AMO(50µM) with 30 µL of nanotube (1 mg/mL), followed by sonication for 2.5 minutes at 22 Watts/cm² (Q700 Sonicator, Qsonica, Newtown, CT). The assembled NP were mixed with 1 mL of cell culture medium and incubated with cells for forty-eight hours without any transfection reagents. For animal experiments, each dose of NP was generated by mixing 7 µL of AMO (50µM) with 105 µL of JBNT (1 mg/mL), followed by sonication for 2.5 minutes. 45.5µL PEG 400 (Sigma) and 25.2µL glucose solution (55% wt./vol) (Sigma) were added to the NP solution (total volume 182.7 µL). Each control dose contained the same components, except for the 7 µL of control anti-miR (50µM).

NP were also generated for tracer experiments using a molecular beacon for GAPDH. 20µl of 20 nM of GAPDH molecular beacon and 60µl of JBNT (1mg/mL) were mixed and diluted with water to 100µl; then sonicated and mixed with 26µL PEG 400 (Sigma) and 14.4µL glucose solution (55% wt./vol) (Sigma); final volume was 140.4 µl. For transmission electron microscopy studies, a 15 µL sample of nanotube or NP solution was mounted on a carbon-coated copper grid (EM Sciences), and stained using a droplet of 2% aqueous uranyl acetate for 30 s. Excess staining agent was blotted with filter paper and the grid was dried at room temperature. The images were obtained on a Morgagni™ 268 microscope (FEI) at a magnification of 20,000 to 140,000 under an acceleration voltage of 80 kV. A toxicity study has been conducted with NPs(15;22). They did not cause apoptosis in vitro or organ toxicity in vivo at the concentrations and doses used in this study.

RNA Isolation.

Total RNA including microRNA was extracted from CS-1 and JJ cells and xenograft tumors using miRNeasy Mini Kit (Qiagen, Valencia, CA). The concentration and quality of total RNA were determined with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Samples with purity of 1.8–2 and integrity over 1.6 were used for analysis of miRNA expression.

microRNA Expression.

Total RNA was reverse transcribed using the miScript Reverse Transcription kit (Qiagen, Valencia, CA), and quantification of the ubiquitously expressed miRNA *U17a* was used as an internal control. A reaction mixture (20 µl) containing the SYBR Green Master Mix (Qiagen), 2 ng of cDNA template plus miScript Universal primer and miScript Primer Assay (miR specific primer for miR-181a) in a 96-well plate was used for real-time PCR using miScript SYBR Green PCR kit (Qiagen). The reactions were done in triplicate on the DNA engine CFX96™ Real-time PCR amplification system (Bio-Rad, Hercules, CA). PCR conditions: an initial step at 95 °C for 10 min, followed by 40 cycles of amplification at 94 °C for 10 s, 55 °C for 30s, then 70°C for 30s.

mRNA Expression.

RGS16 and *MMP1* mRNA were quantified using the Reverse Transcription System (Bio-Rad) followed by real-time PCR with SYBR Green Master Mix (Qiagen). *B2M* was used as the internal control(23;24). The primers for *RGS16*, *MMP1*, and *B2M* have been previously published (25–27). The comparative threshold cycle (Ct) method, i.e., 2-ΔΔCt method was used for the calculation of fold amplification(28). The data analysis was performed as previously described(26;28).

ELISA Assay.

Lysates from conditioned media (CM) from cultured cells and homogenized xenograft tumors were used for ELISA assay. Cells were cultured for one day, then the medium was changed to 1% FBS O/N, and the CM were collected to measure VEGF and pro-MMP1 concentration (R&D system, Minneapolis, MN)(20;25). Each sample was measured in duplicate and each experiment was repeated at least 3 times. VEGF and MMP1 were normalized to the lysate protein concentration as determined by Quick Start Bradford protein assay (Bio-Rad, Hercules, CA).

Mouse model, bioimaging, tumor growth, and metastasis analysis.

Xenograft tumors in nude mice were generated as previously described(20). Briefly, 1×10^6 cells in 100 µL culture medium mixed with 300 µL Matrigel™ (BD Biosciences, San Jose, CA) were injected subcutaneously in the back of nude mice (nu/nu 6–8-week-old, female, Charles River Laboratory, Wilmington, MA).

In vivo bioimaging was performed with Fluorescence Molecular Tomography (FMT, PerkinElmer, Waltham, MA) two weeks after the start of treatment. Twenty-four hours before imaging, mice were injected via tail vein with 2 nmol MMPSense 680 and

AngioSense 750 (PerkinElmer, Waltham, MA). Mice were anesthetized with ketamine (i.p.) during FMT imaging. FMT is acquired with a continuous wave-type scanner capable of acquiring transillumination, reflectance, and absorption data at 680 nm excitation and 700 nm emission or 750 nm excitation and 780 nm emission (PerkinElmer). AngioSense and MMPsense content in xenograft tumors was determined by region of interest analysis as previously described (20).

Mice were treated with seven IV injections of NP^{anti-miR-181a} or control over a three-week period starting two weeks after implantation of chondrosarcoma cells. Tumors and lungs were harvested at 6 weeks after implantation of tumor cells or sooner if required by our IACUC protocol as determined by veterinary staff, who were blinded to treatment group.

Primary tumor analysis.

Tumor size was measured throughout the experiment and tumor weight was determined at the time of excision. Tumor volume was calculated by the formula $V = H \times W \times L \times 0.52$, where H, W, L are the height, width, and length of the tumor, respectively. Part of the excised tumor was fixed in 10% formalin overnight, paraffin embedded, and used for H&E staining. Some of the tumor was stored in RNAlater for RNA extraction or lysis buffer for protein extraction.

Metastasis Analysis.

The number of mice in each group with metastases were compared with the Chi-Square test. Lung metastatic burden was quantified as previously described(29). Briefly, lungs were analyzed with microscopy after fixation in 10% formalin. Transverse sections were made at 350 μ m intervals yielding approximately 40 sections per lung. Hematoxylin and eosin stained slides were scanned using the Philips Ultra Fast Scanner (Philips, Amsterdam, Netherlands) and extracted images were analyzed using Image Pro image analysis software (Media Cybernetics, Rockville, MD). Lung tissue area was measured using an automated algorithm. To correct for variation in bronchus dilation, subtraction of dead space of the larger airways and vascular spaces was included as a component of the algorithm. Measurement of the area of the metastases, as well as exclusion of non-pulmonary tissues, was performed manually. Metastatic burden was quantified as the proportion of sections with metastases, the total number of nodules per lung, and the total area of the nodules normalized to the total lung area. The average area fraction is an accepted estimate of the volume fraction(30).

Study approval.

All animal studies were approved by the IACUC at Rhode Island Hospital and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, (eighth edition).

Statistics.

All experiments were repeated at least 3 times. Experiments with two groups were analyzed with the Student's t-test unless otherwise specified (Mann-Whitney U test was used for data not normally distributed). Experiments with three or more groups were compared with one-

way ANOVA, followed by the Student's t-test with Bonferroni correction for individual comparisons. Data are presented as means $\pm$ SD.

Generalized linear models were used to compare mice who received anti-miR-181a to control anti-miR. The negative binomial distribution was used for analysis of MMPsense and AngioSense probe content, tumor weight, and total number of nodules per lung. The negative binomial distribution is positively skewed with distinct parameters for central tendency and variance, as well as having no negative values. These variables are each positively skewed and cannot be negative. This makes the negative binomial distribution superior to the Gaussian distribution for these measures. The binomial distribution was used for analysis of the proportion of sections with metastases and the normalized areas of nodules. The binomial distribution represents a distribution with parameters for a rate of event within a number of opportunities which more appropriately bounds the statistical model to fall between zero and 100, as well as appropriately skewing the distribution towards 50% as estimates approach the bounds. Classical sandwich estimation was used to adjust for any model misspecification. The Wilcoxon weighted chi-square test was used to compare Kaplan-Meier survival functions of times-to-event outcomes. Time was measured in days from tumor implantation. Statistical analysis was performed with SAS version 9.4 (The SAS Institute, Cary, NC) and Prism version 5.04 (GraphPad, San Diego, CA). The null hypothesis of no difference was rejected at a significance level of 5%.

Results

Nanopiece delivery of nucleotides

Janus base nanotubes (JBNT) (Fig. 1B, left panel) were mixed with AMO and NP (Fig. 1B, right panel) were created as described in the Methods section. Transmission electron microscopy was used to analyze JBNT and NP. The JBNT were too long and intertwined to measure, whereas the average size of NP is $112.3\text{nm} \pm 48.6\text{nm}$ in length and $17.3\text{nm} \pm 9.5$ in width (Fig. 1B, right panel). As a first step to test if NP can deliver AMO to chondrosarcoma cells, we incubated CS-1 cells with Cy3- labeled control AMO alone or with NP^{AMO}. Intracellular fluorescence is only seen with NP^{AMO} (Fig. 1C, upper left) and not with AMO alone (Fig. 1C, lower left). To test if microRNA knockdown could be achieved with NP delivery, CS-1 and JJ cells were incubated with NP^{panti-miR-181a}. NP^{panti-miR-181a} reduced expression of miR-181a (Fig. 1D), restored expression of RGS16 (Fig. 1E) and reduced further downstream targets VEGF and MMP1 (Fig. 1F, G) compared to NP^{control AMO}.

To determine whether NP can deliver nucleotide sequences intracellularly in vivo, a molecular beacon for GAPDH (mbGAPDH) alone or carried by NP was administered by tail vein injection to mice bearing xenograft tumors. The molecular beacon contains a nucleotide sequence complementary to a specific mRNA target flanked by a quencher and a fluorescent probe, which fluoresces only after binding the intracellular target mRNA, in this case a house keeping gene GAPDH. Tumor fluorescence was only observed when the NP^{mbGAPDH} was used (Fig. 2). Fluorescence is also seen in the spleen and liver in both the control and experimental groups, indicating that the molecular beacon can be taken up by the mononuclear phagocyte system directly. In contrast, mbGAPDH signals were only seen in

the tumor when NP was used for delivery, indicating NP was required for beacon delivery to the tumor.

We then compared local tumor injection to systemic injection of NP^{panti-miR-181a}. miR-181a expression was reduced by 52% when xenograft tumors were injected with NP^{panti-miR-181a} (Fig. 3A). Similarly, when NP^{antagomir-181a} (at doses of 0.35 and 0.7 nmol of anti-miR) were administered systemically, miR-181a was reduced to 44.7% and 32.2% of control respectively (Fig. 3B). In subsequent *in vivo* experiments the lower dose was used for tail vein injections because of limited availability of JBNT.

Nanopiece/anti-miR-181a inhibits miR-181a expression, tumor growth, MMP1 expression and activity, and increases survival in a xenograft model.

A mouse xenograft chondrosarcoma model was used to assess whether NP could be used as a delivery platform for nucleotide based therapy. MiR-181a was reduced to 46% of control in the xenograft tumors (Fig. 4A). In prior work, we found that RGS16 is a direct target of miR-181a, and that diminished expression of RGS16 enhances CXCR4 signaling, which culminates in MMP1 and VEGF expression(11). In the treated mice, RGS16 mRNA expression was restored and MMP1 mRNA expression decreased (Fig. 4B) and both MMP1 and VEGF protein content decreased (Fig. 4C). Florescence Molecular Tomography (FMT) *in vivo* imaging indicated decreased MMP activity in the tumors (Fig. 4D); angiogenesis was not significantly affected (Fig. 4E). Tumor weight was reduced by 25% (Fig. 4F) and tumor volume by 32% at day 38 (Fig. 4G). More importantly, survival as measured by days to forced euthanasia was almost doubled at day 48 in the treatment group (23% vs 45%) (Fig. 4H). There were fewer mice with metastases (11/13 vs 5/11, $p<0.04$) and a decrease in the proportion of lung sections with tumor (0.154 vs 0.048) (Fig. 4I, J). The number of metastatic nodules per lung (8.4 vs 2.4) and normalized area of nodules per lung (0.12 vs 0.04) were not different (Supplemental Figure 1). Taken together, the results indicate that the systemic delivery of NP^{panti-miR-181a} inhibited miR-181a overexpression, downstream targets, and tumor progression.

Discussion

We previously showed as proof of principle that miR-181a is a therapeutic target by pre-transfection of CS-1 cells with a lenti-virus expressing anti-miR-181a before implantation into mice(11). This reduced miR-181a in xenograft tumors, and inhibited CXCR4 signaling and tumor progression(11). In this study, we used a non-viral, biomimetic nanoparticle to deliver AMOs. Essentially all the effects achieved with the lenti-virus construct were recapitulated here, albeit to a lesser degree, suggesting that our NP delivery platform is successful for delivering AMOs in a preclinical model. In addition, survival was improved in the current study. Most if not all advanced cancers are incurable, and current targeted therapies aim to slow tumor progression and improve quality of life.

In prior work we determined that one mechanism of miR-181a overexpression that results in tumor metastasis is inhibition of RGS16(11). RGS proteins are critical modulators of signal transduction pathways in normal physiology and in cancer. Diminished RGS16 leads to progression of several types of carcinoma as well as chondrosarcoma(11;31–33). RGS16 is

an innate negative regulator of CXCR4 signaling, so that inhibiting expression of an inhibitor results in gain of function in CXCR4 signaling. CXCR4 expression is increased in chondrosarcoma and mediates cell motility and metastasis(20;25). CXCR4 signaling activity is enhanced by inhibition of RGS16, which results from increased miR-181a expression. Increased miR-181a and CXCR4 expression are at least partially a result of hypoxia(11;25). Increased CXCR4 signaling increases MMP1 and VEGF expression, invasion and metastases(10;11;20;25). Angiogenesis and invasion are partially mediated by VEGF and MMP, are enhanced by hypoxia, and are critical features of malignancy(34). Our work has focused on understanding these aspects of tumor biology and developing targeted therapeutics. Based on the effects of knocking down miR-181a expression with a lenti-virus construct on RGS16 expression and inhibiting metastatic pathways, we postulated that antagomir based therapy might be efficacious and potentially better than inhibition of CXCR4 signalling with the drug AMD3100(11;20). The advantage of the AMO approach is that multiple, partially redundant signaling pathways related to tumor progression may be targeted; one reason AMO strategies are under clinical development (35;36). Another potential advantage of NPs is that they can be loaded with combinations of shRNA, RNAi, AMO, and drugs(37;38). In this study, we demonstrated that an AMO strategy can inhibit expression of an oncomiR (miR-181a), restore RGS16 expression (Fig. 4B), inhibit tumor progression, and prolong survival. Therefore, NP^{AMO} may be an effective treatment for chondrosarcoma, other tumor types, and diseases of cartilage in which RGS16 and other miR-181a targets are overexpressed. The twice-weekly systemic administration that was used in this study could be reasonably utilized in the clinic. In comparison to AMD3100, which was administered b.i.d for 6 weeks, and continuous knockdown of miR-181a with the lenti-virus construct, the effects on tumor growth and metastases were, as expected, less in this study.

There are several technical challenges in the use of nanoparticles in clinical applications. One challenge is penetration of tumor tissue with therapeutics. Similar to chondrocytes, chondrosarcoma cells produce a cartilaginous matrix which is composed of type II collagen and proteoglycans. Proteoglycans are negatively charged and the pore size in cartilaginous matrix ranges from 6 to 100 nm(39). These properties may limit anti-cancer drug diffusion and infiltration.(40). An important property of NP is their positive surface charge. After ultrasonic treatment, NP^{AMO} are rod shaped and have a diameter of only 17nm, much smaller than conventional spherical nanoparticles which have diameters typically larger than 60nm. (Fig. 1A) (40–43). The positive charge and small size are advantageous in penetration of the matrix and we have also shown that NP can penetrate articular cartilage matrix(16).

Toxicity is of concern with clinical use of nanoparticles. Viruses can cause inactivating immune responses and have mutagenic risks(44). Lipid nanoparticles have been used for tumor delivery of drugs and RNA therapeutics. However, a recent Phase 1 clinical trial for solid tumor treatment utilizing MRX34, a liposomal miR-34a mimic, was terminated due to unexplained immune related serious adverse events. It is not clear if the toxicity was related to miR34a mimic or the liposomal nanoparticles(45). An additional advantage of NP is their low toxicity and better safety profile compared to viruses or lipid based nanoparticles, since they are assembled from nucleotide derived JBAK that mimic natural biological molecules(16).

There are some limitations to our study. We did not attempt to target NP to the tumor cells but relied on increased vascular permeability characteristic of tumor vessels. Nontargeted NP therapy is similar to the delivery of chemotherapy in other cancers. We did not determine the mechanism of NP uptake by tumor cells, although we think it may be mediated by endocytosis.

While we identified RGS16 as one relevant target of miR-181a, in this study, we did not attempt to validate other targets of miR-181a. We have however, identified additional candidate targets by comparing CS-1 cells transfected with control miR or anti- miR-181a with gene array analysis. These data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE70065 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70065>)(46). We used a subcutaneous xenograft model rather than an orthotopic model. An advantage of the subcutaneous model is more consistent tumor growth kinetics, more precise measurement of tumor growth, and better in vivo bioimaging. An advantage of the orthotopic model is tumor – stromal cell interaction, which can facilitate tumor progression. A disadvantage is the functional impairment and distress to the animals which can limit the length of the experiments and survival analysis.

In conclusion, our results confirm that miR-181a is an oncomir, whose knockdown via systemic delivery restores expression of RGS16 and inhibits tumor progression. Further optimization of formulation, targeting, dosing, and loading of NP with AMO or combinations of different microRNAs, AMOs, and drugs may yield better results. NP is a nonviral platform for experimental delivery of nucleotide based therapeutics and AMO therapy that may have potential for cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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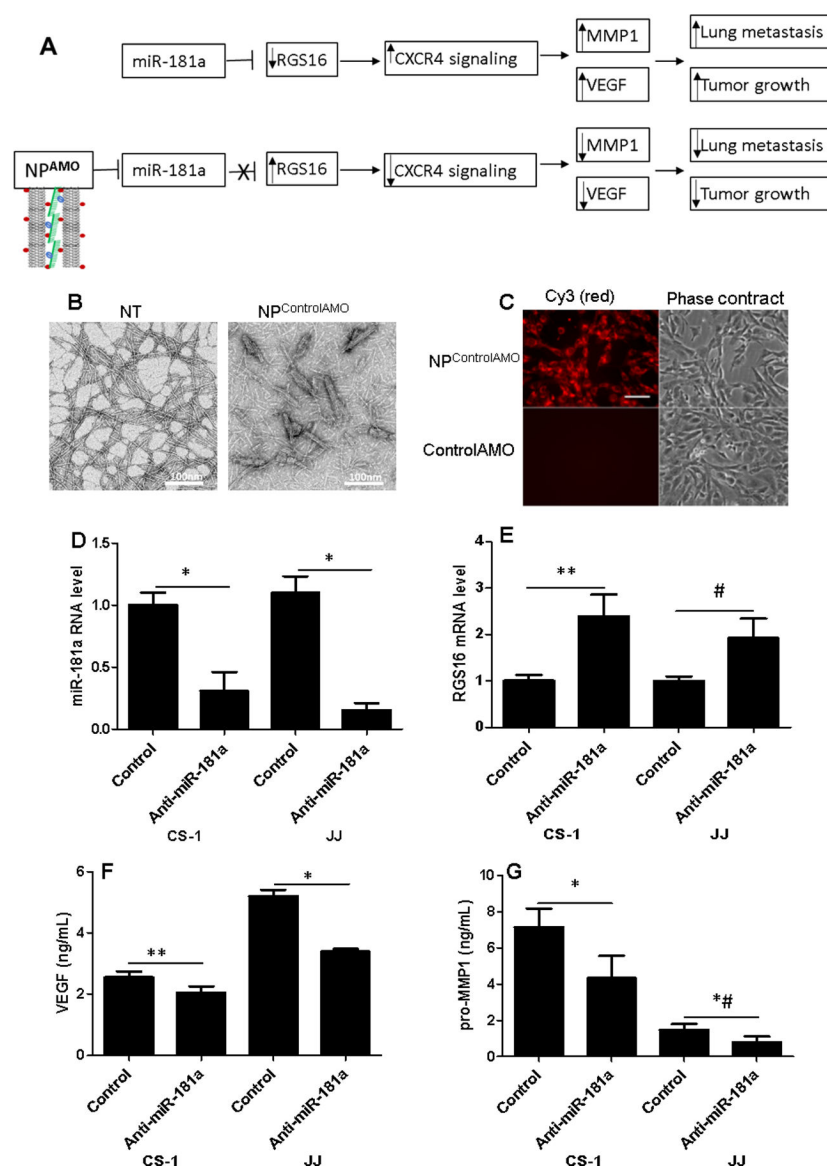


Figure 1. Nanopiece characterization and transfection of chondrosarcoma cells. (A) (Upper) Pathway by which endogenous overexpression of miR-181a enhances CXCR4 signaling via inhibition of RGS16 is shown. CXCR signaling increases MMP1 and VEGF expression, leading to tumor progression. (Lower) Schematic of anti-miR-181a (AMO) delivery by NP to chondrosarcoma cells and reversal of enhanced CXCR4 signaling, resulting in inhibition of tumor progression. Red indicates positive charges on NP, blue indicates negative charges on AMO (green). (B) Transmission electron microscopy of Janus base nanotubes (JBNT) (left) and Nanopieces combined with AMO (right). After combination of JBNT with AMO and ultrasonic treatment, the size was reduced. Scale bar, 100 μm. (C) Transfection with NP was first evaluated using Cy3 -labeled control AMO. Representative images one day after incubation with NP^{Control-Cy3} (upper panel) or Control-Cy3 alone (lower panel). Scale bar, 50 μm. (D) miR-181a expression was evaluated with real-time PCR two days after incubation with NP^{anti-miR-181a} or control (* p<0.001, n=3). (E)

RGS16 mRNA was quantitated with real-time PCR after transfection with NP^{anti-miR-181a} or control (** $p < 0.01$, #, $p < 0.05$, $n = 3$). VEGF (F) and pro-MMP1(G) were quantified in conditioned media with ELISA after transfection with NP^{anti-miR-181a} or control (** $p < 0.01$, *, $p < 0.001$, #, $p < 0.05$, $n = 4$).

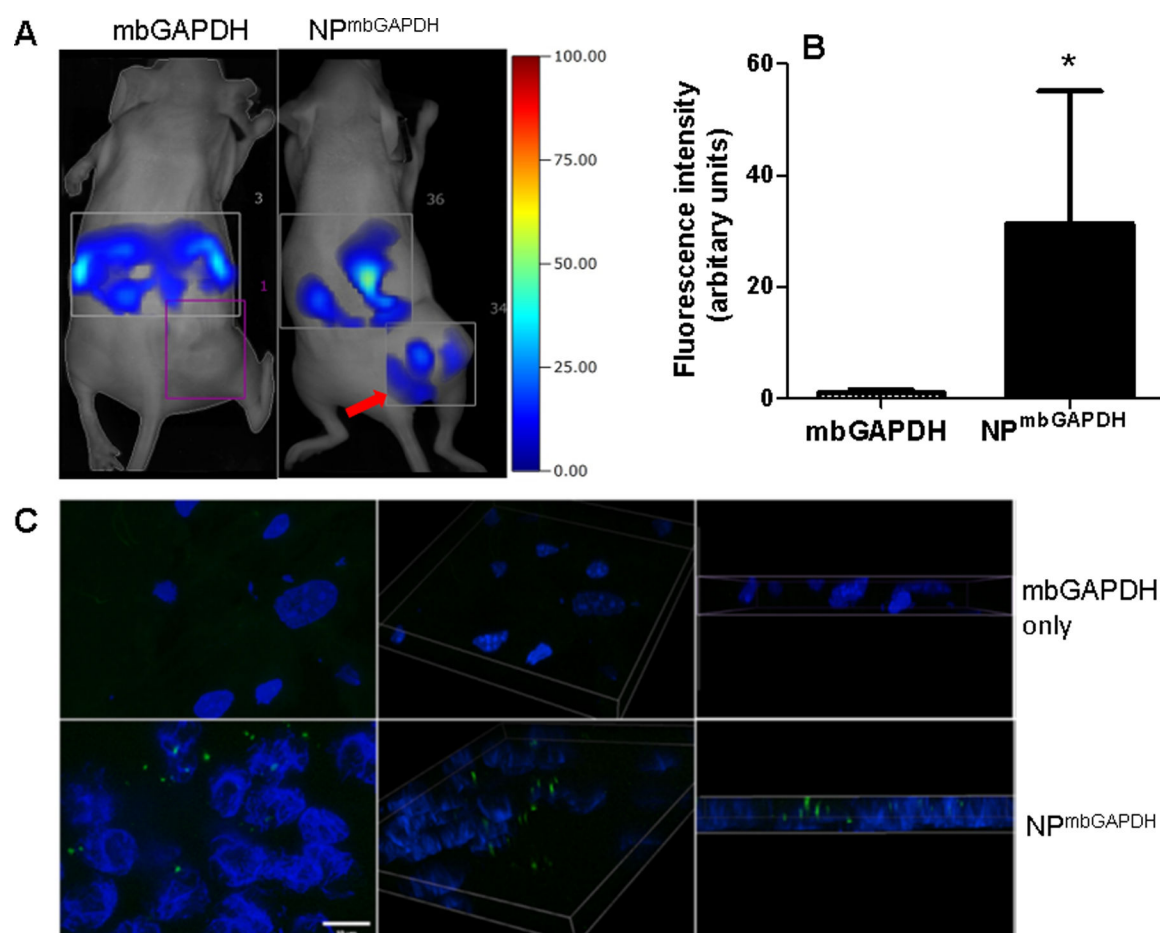


Figure 2. Nanopieces transfected xenograft tumors with molecular beacon.

Mice bearing xenograft tumors were administered GAPDH molecular beacon alone (mbGAPDH) or with NP (NP^{mbGAPDH}). (A) FMT images one day later show tumor fluorescence only after combination of beacon and NP (arrow). (B) Quantification of Fluorescent intensity in xenograft tumors (n=4, 5; *, p<0.02, Mann-Whitney U). (C) Confocal microscopy of xenograft tumor sections demonstrate intracellular fluorescence after NP^{mbGAPDH} (lower panel), but not with mbGAPDH alone upper panel). Size bar = 10μm.

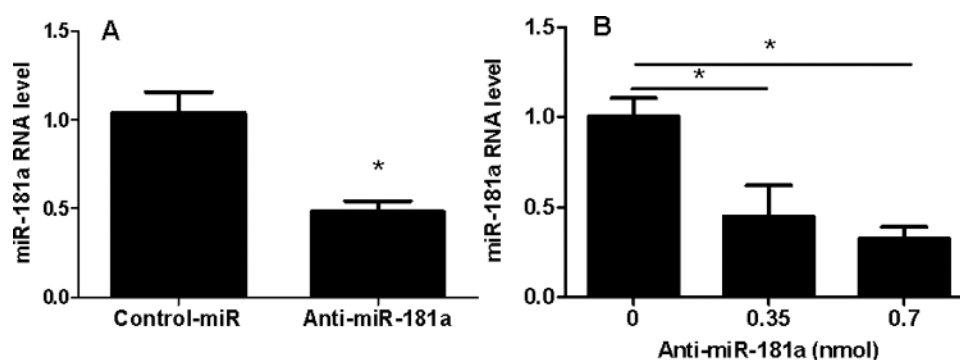


Figure 3. Both local and systemic delivery of NP^{anti-miR-181a} reduce miR-181a expression in xenograft tumors.

(A) miR-181a in xenograft tumors was quantified with real-time PCR two days after local injection of NP-control AMO or NP-anti-miR-181a (0.35 nmol) into xenograft tumor two times/week for two weeks. *, $p < 0.001$, $n = 8$. (B) miR-181a level in xenograft tumors two days after systemic administration of three doses of NP-control AMO or NP-anti-miR-181a (*, $p < 0.001$, $n = 3/\text{group}$).

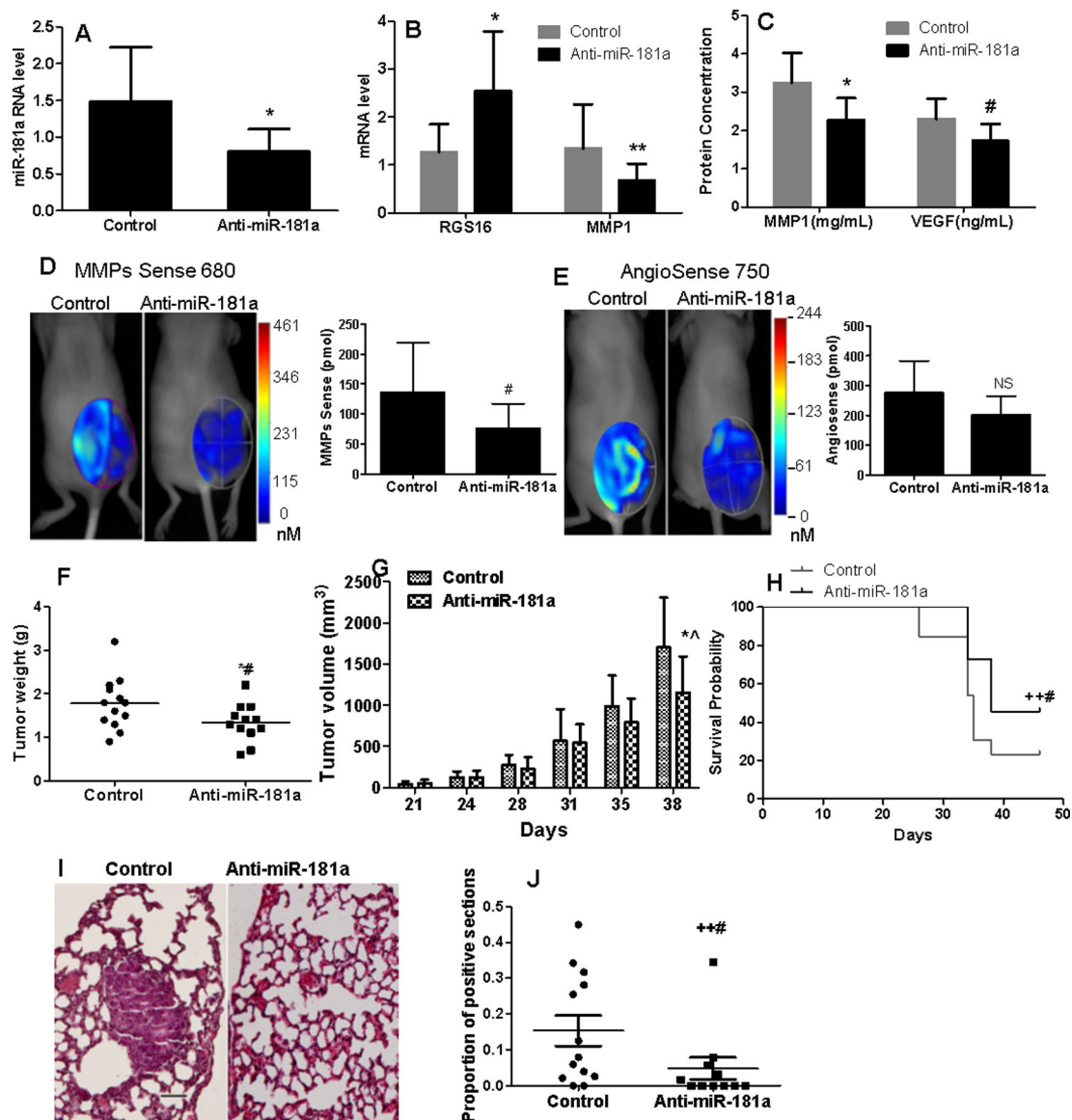


Figure 4. Systemic NPanti-miR-181a restores RGS16 expression and inhibits chondrosarcoma growth.

Mice bearing xenograft tumors were treated with seven IV injections of NP-control AMO or NP-anti-miR-181a over a three-week period starting two weeks after implantation of chondrosarcoma cells. (A) qRT-PCR quantification of miR-181a in xenograft tumors. (*, $p < 0.01$, $n = 11$ /group Mann-Whitney U). (B) qRT-PCR quantification of *RGS16* and *MMP1* mRNA level. (*, $p < 0.01$, Mann-Whitney U; **, $p < 0.04$, $n = 11$ /group). (C) MMP1 and VEGF protein expression in xenograft tumors. (*, $p < 0.01$; #, $p < 0.03$, $n = 8$ /group). (D&E) FMT imaging with MMPs Sense and AngioSense ($n = 13$, 11, #, $p < 0.03$; NS, $p < 0.06$). (F) Final tumor weight (*#, $p < 0.05$). $n = 13$, 11. (G) Tumor volume (*#, $p < 0.02$, $n = 13$, 11). (H) Kaplan-Meier Survival Curves (##, $p = 0.05$, $n = 13$, 11). (I) Representative H&E sections of lungs from control and treatment groups. Metastatic burden (J) Proportion of positive sections (##, $p = 0.05$, $n = 13$, 11).