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MicroRNA-Regulated Non-Viral Vectors with Improved Tumor Specificity in an Orthotopic Rat Model of Hepatocellular Carcinoma

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Abstract

In hepatocellular carcinoma, tumor specificity of gene therapy is of utmost importance to preserve liver function. MicroRNAs are powerful negative regulators of gene expression and many are down-regulated in human HCC. We identified seven miRNAs that are also down-regulated in tumors in a rat hepatoma model ($p<0.05$) and attempted to improve tumor specificity by constructing a panel of luciferase-expressing vectors containing binding sites for these microRNAs. Attenuation of luciferase expression by the corresponding microRNAs was confirmed across various cell lines and in mouse liver. We then tested our vectors in tumor-bearing rats and identified two microRNAs, miR-26a and miR-122, that significantly decreased expression in liver compared to control vector (6.40% and 0.26%, respectively; $p<0.05$). In tumor, miR-122 had a non-significant trend towards decreased (~50%) expression , while miR-26 had no significant effect on tumor expression. To our knowledge this is the first work using differentially expressed microRNAs to de-target transgene expression in an orthotopic hepatoma model and identification of miR-26a in addition to miR-122 for de-targeting liver. Considering the heterogeneity of microRNA expression in human HCC, this information will be important in guiding development of more personalized vectors for the treatment of this devastating disease.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

Supplementary information is available at Gene Therapy's website.

Keywords

hepatocellular carcinoma; bioluminescence; microRNA; rat model; gene therapy

Introduction

Hepatocellular carcinoma (HCC) is the 5th most common malignancy and the 3rd leading cause of cancer-related death worldwide [1]. Moreover, the incidence of HCC is continuing to rise, most likely due to continued rise in the prevalence of risk factors such as Hepatitis B and C infection and increased alcohol consumption in industrialized countries [1]. HCC is often detected at very advanced stages with limited treatment options. While surgical resection and liver transplantation are the best curative options, only a small proportion (<20%) of patients are candidates for such procedures. Hence, new therapies are urgently needed to stem the overwhelming mortality associated with this deadly disease.

MicroRNAs (miRNA) are a large class of highly conserved, short (~22 nucleotide; nt), non-coding RNAs that bind to imperfectly complementary sequences in the 3'-untranslated regions (3'-UTR) of target mRNAs [2]. In many species including humans, upon binding to mRNAs, miRNAs modulate protein production via either translational repression or mRNA degradation [3]. The regulatory mechanism employed is primarily related to the degree of complementarity of the mRNA:miRNA duplex, with full complementarity leading primarily to mRNA cleavage and degradation, similar to small interfering RNAs [1,4]. In humans, approximately 30% of mRNAs are regulated by miRNAs and each miRNA is able to regulate a large number (100s) of mRNAs [1,5,6]. As such, miRNAs are critical biological regulators during development and differentiation, and their deregulation has been linked to the pathogenesis of many diseases including cancer [1,2,7,8]. In HCC, many miRNAs are aberrantly expressed [2,3,9–11], leading to de-regulated expression of proteins involved in HCC development [1,3,12], and highlighting their potential as biomarkers for improved HCC diagnosis and prognosis, and as therapeutic targets for HCC gene therapy [1,10,13–17].

Both viral and non-viral therapeutic gene transfer has been explored as an alternative and attractive therapy for HCC [1,2,4,18]. The challenge of these therapies is to express high levels of therapeutic transgene within tumors in order to maximize tumor kill while minimizing expression in normal tissues, particularly the liver, to maximize safety. Recently, in an elegant set of studies, Brown et al were the first to develop a new targeting approach exploiting endogenously expressed miRNAs called "transcriptional de-targeting" [2,3,19–21]. By incorporating perfectly complementary miRNA target sites (miRTs) into vectors, these studies showed that it was possible to de-target expression from cells containing high levels of the corresponding miRNA and restrict expression to cells lacking the miRNA. Since miRNA expression levels in cancer cells compared to normal cells are frequently attenuated [3,4,22], several groups have explored this exciting strategy to improve tumor specificity of therapeutic or oncolytic viruses [1,4–6,23–29].

In this study we explored the use of transcriptional de-targeting of non-viral expression vectors for improving the tumor specificity of transgene expression in an orthotopic rat model of HCC (Figure 1). Our goals were to assess the expression levels of relevant

miRNAs in both rat HCC and liver, develop imaging expression vectors regulated by miRNAs down-regulated in HCC, and explore the ability of these miRNA-regulated vectors to improve the tumor specificity of transgene expression in living subjects.

Results

Several microRNAs are expressed at lower levels in both human HCC and a rat HCC model compared to normal liver

Our first goal was to identify microRNAs that were expressed lower in rat and human HCC versus normal liver. Rather than perform a broad search (e.g., microarray) for target miRNAs we focused on miRNAs that have literature support for their lowered expression in human HCC versus normal human liver and therefore our microRNA-regulated strategy should have the highest translational potential. The following 11 microRNAs were identified as potential targets: miR-23a [1,5–8,11]; miR-26a [1,2,7–11,16]; miR-101a [2,9–11,30]; miR-122 [1,3,12,31,32]; miR-125a-5p [31]; miR-125b [31]; miR-130a [31]; miR139-5p [33]; miR-150 [32]; miR-199a-5p [12,32]; and miR-223 [32]. Expression levels of these miRNAs were determined from both rat liver (n=8) and rat HCC (n=5) two weeks following tumor cell implantation (Figure 2A). As expected miR-122, a liver-specific miRNA [34], was expressed at the highest level in liver and all data was normalized to this value. Seven of the 11 miRNAs examined were significantly decreased in tumor versus liver ($p<0.05$) including: miR-26a (5.47-fold); miR-101a (3.93-fold); miR-122 (13.23-fold); miR-125a-5p (2.39-fold); miR-125b (8.85-fold); miR-139-5p (16.67-fold); and miR-150 (4.96-fold). None of the miRNAs were expressed at higher levels in tumor versus rat liver.

miRNA-regulated vectors targeted to different miRNAs are differentially expressed across various cell lines and can attenuate liver expression *in vivo*

Based on the above expression results we constructed several plasmids expressing the firefly luciferase (Luc2) gene driven by a CMV promoter (pCMV) to carry 4 tandem sense or antisense (Anti-) target sites (miRTs) to the following miRNAs within the 3' untranslated region (3' UTR): miRT-26a (26a); miRT-122 (122); miRT-139-5p (139-5p); miRT-150 (150); miRT-199a (199a), Anti-miRT-26a (Anti-26a), Anti-miRT-122 (Anti-122) (Figure 2B). The original vector carrying no miRTs (No miRT) was used as a control. Several of these vectors were first tested for their ability to be regulated by miRNAs in HUH-7 (human hepatoma cells), McA-RH7777 (rat hepatoma cells) and 293T (human embryonic kidney cells). These cell lines were chosen for testing since they express variable levels of miR-122 and miR-26a (Figure 3A). HUH-7 cells expressed a high level of miR-122 and relatively lower level of miR-26a, McA-RH7777 cells expressed a moderate level of miR-26a and lower level of miR-122, and 293T cells expressed low levels of both miRNAs.

Each cell line was transfected with one of the above Fluc expressing vectors and a humanized renilla luciferase (hRluc) expressing vector (pCMV-hRluc) to control for transfection efficiency. In all cases, except one (Anti-26a in HUH-7 cells), the lengthening of the 3' UTR by inclusion of anti-sense miRNA binding sites significantly decreased Fluc activity by an overall average of 22.3% ± 15.0% across all cell lines ($p<0.05$) (Figure 3B–D). Importantly, in HUH-7 cells where miR-122 is expressed at high levels, the 122 vector

showed the lowest amount of Fluc activity (Figure 3B), and in McA-RH7777 cells where miR-26a is expressed at higher levels, the 26a vector showed the lowest amount of Fluc activity (Figure 3C). No significant difference in Fluc activity was seen in 293T cells between the sense and anti-sense vectors (Figure 3D).

Next, the No miRT, Anti-122 and 122 vectors were tested *in vivo* in normal Balb/C mice following liver targeting via hydrodynamic injection [35]. This vector was chosen since miR-122 accounts for approximately 70% of all miRNAs expressed in the liver [34]. Forty-eight hours after vector delivery, bioluminescence imaging was performed and regions of interest over the liver were analyzed for Fluc activity (Figure 4A). As shown in Figure 4B, in contrast to the *in vitro* results the Anti-122 vector showed similar levels of Fluc activity within the liver compared to the No miRT vector. Importantly, the 122 vector showed almost two orders of magnitude lower Fluc activity compared to both other control vectors (1.15% versus No miRT; $p<0.05$).

Differentially expressed microRNAs can be used to attenuate liver expression but maintain tumor expression in a rat model of HCC

Finally, we tested seven of our constructs in our rat model of HCC since this model showed differential regulation of miRNAs between normal liver and tumor similar to human HCC. In our experience with this model a single solitary tumor approximately 200–1000 mm^3 in size develops over the course of two weeks post-cell implantation (ultrasound measurements; unpublished data). At this time point we tested the No miRT, 122, 26a, 139-5p, 150, and 199 vectors. The No miRT and 199 vectors both served as controls since miR-199 was found to be expressed at extremely low levels in both liver and tumor (Figure 2A). Each Fluc vector (400 μg) was hydrodynamically injected into tumor-bearing rats along with our Rluc vector (100 μg) to control for delivery. Both liver and tumor were harvested 48 hours after injection, and Fluc activity, Rluc activity and protein levels were determined to assess normalized Fluc activity. Five rats also received a mock injection of PBS to determine the background level of Rluc activity and identify tissues that were transfected and further analyzed (i.e., any tissue expressing Rluc above background level was further analyzed for Fluc and protein). Within liver lysates, all rats receiving no miRT or miRT vectors (n=31) had Rluc values above background values from PBS injected animals (Supplementary Figure 1A). As expected, tumor Rluc values were much lower since hydrodynamic injection primarily targets the liver; however, 20 of the 31 tumors showed Rluc values above background and were used for analysis (Supplementary Figure 1B). Absolute normalized Fluc expression levels were approximately 100-fold higher in liver than tumor, which may reflect differences in expression from the CMV promoter between these two tissues types (Figure 5A). For this reason, relative comparisons between No miRT vector and all other vectors were made within liver or tumor (Figure 5B and C). In both liver and tumor no difference in Fluc activity was detectable between the No miRT and 199 control vectors (Figure 5B and C). Importantly, within the liver both the 122 and 26a vectors had significantly lower Fluc expression ($p<0.05$) compared to both control vectors (Figure 5B). In striking contrast these same vectors did not result in significant repression of Fluc activity within the tumor (although 122 vector showed a trend towards decreased expression), indicating that vectors regulated by miR-122 and miR-26a can help attenuate

off-target expression in the liver but maintain high expression levels in the tumor (Figure 5C).

Discussion

The primary goal of gene therapy is to genetically-modify a chosen population of target cells while limiting or eliminating off-target expression in normal cells. In cancer gene therapy the goal is to express therapeutic genes specifically within cancer cells so that unwanted toxicity in normal tissues is prevented. In most HCC patients, normal liver health is already compromised so limiting liver expression is of utmost importance. However, one also wants to maintain high levels of expression in tumor tissue to maximize tumor kill. This has proven a difficult task since most strategies that have high tumor specificity also tend to have low tumor expression and vice versa. By leveraging on the differential expression of miRNAs between liver and tumor we have identified 2 miRNAs, miR-122 and miR-26a, that are down-regulated in both our rat model of HCC and human HCC, and have shown that miRNA-sensitive vectors towards these 2 miRNAs can selectively "de-target" transgene expression in normal liver but maintain high expression in HCC.

Several groups have used miRNAs to "de-target" therapeutic or oncolytic viruses [23–29,36–39]. Jin et al (2011) recently showed that incorporation of 8 imperfect Let-7 sites downstream of the E1A gene can limit an oncolytic adenovirus's replicative capacity within normal liver cells but maintain it in HCC cell lines with low Let-7 expression [27]. Intratumoral injection of this virus into Let-7-expressing subcutaneous HCC xenografts resulted in significantly lower viral replication but in HCC xenografts with low Let-7 expression tumor growth was significantly retarded. Therefore, this vector has therapeutic potential in patients harboring Let-7-negative tumors. However, this study also showed that Let-7 expression was equivalent or even higher in the majority (63.6%) of HCC tumors compared to normal liver. Therefore, in patients harboring Let-7 positive HCC, theoretically this Let-7-sensitive vector would not be efficacious since viral replication would be limited. This highlights the need to identify additional miRNAs, such as miR-122 and miR-26a, that can be used to selectively control transgene expression across multiple HCC subtypes. As with Let-7 expression, miR-122 and miR-26a down-regulation is not a rule in HCC [9,16]. Due to this heterogeneity in miRNA expression across HCC tumors with varying etiology, one can imagine development of a suite of "personalized" therapeutic vectors regulated by one or more miRNAs (miR-122, miR-26a, Let-7, etc.) that would be administered to individual patients based on the miRNA transcriptome (miRNome) of their tumor(s) and liver. Future work identifying miRNAs that are lost in a particular subtype of HCC will be of tremendous value towards reaching this goal [40].

One limitation of the miRNA-de-targeting strategy is that expression is never completely suppressed in a population of cells expressing a chosen miRNA. This is true even for highly expressed tissue-specific miRNAs, such as miR-122 in the liver, as we demonstrated in both mice (Figure 4) and rats (Figure 5). Therefore in the future, to achieve complete elimination of off-target expression we plan to combine miRNA-regulation with other targeting strategies. Several strategies have been proposed to achieve improved tissue specificity of gene therapy vectors including augmenting vector tropism towards cancer cells [41], localized

intratumoral [42] [43] or intraarterial administration [44], and tissue-specific regulation of transgene expression [43,45,46]. Fu et al. recently pursued this latter type of strategy by combining the use of the liver-specific apolipoprotein E (apoE)-AAT promoter with miR-122a regulation to restrict replication of an oncolytic herpes simplex virus in HCC xenografts after intratumoral injection [28]. We have recently developed a transcriptionally-regulated, amplifiable adenoviral vector driven by the tumor-specific Survivin promoter that shows superior tumor specificity in our orthotopic rat model of HCC following systemic administration compared to vectors driven by the strong, constitutive CMV promoter [46]. This virus also utilized the two-step transcriptional amplification (TSTA) system that we validated which amplifies expression from weak promoters. Notably, our Survivin-TSTA viral vector was able to show tumor expression levels comparable to that of the CMV promoter-driven vector [46]. However, as with most tumor-specific promoters, our vector did show some background “leaky” expression within the liver, which was most likely amplified by our TSTA system [46]. Therefore incorporating miRNA binding sites into this vector could alleviate this off-target expression to achieve the coveted tumor-high/liver-off profile. In addition it has been shown that different miRNAs can work cooperatively to abrogate expression even further [47], and so combinations of miRTs towards miR-122, miR-26a, and Let-7 should be explored. If this strategy is pursued it will be important to limit the total number of miRTs, since increasing the length of the 3’ UTR may have unwanted effects of expression levels.

As highlighted recently, the degree of miRNA repression is dictated by both miRNA level and mRNA transcript level [48], therefore the amount of miRNA repression in both liver and tumor of vectors driven by strong promoters will be dictated predominantly by the delivery method. Hydrodynamic delivery of vectors primarily delivers constructs to the liver with minimal delivery to the tumor [35]. We confirmed these results since in many tumors we found Rluc expression from our delivery control vector (pCMV-hRluc) was below background levels. Therefore delivering the constructs hydrodynamically favors increased repression in the tumor compared to the liver since mRNA transcript levels will be lower in the tumor than the liver. Strikingly, we saw the opposite result; higher repression in the liver and no significant repression in the tumor. This is because miR-122 and miR-26a are expressed at significantly lower levels in tumor compared to liver to a degree that it overcomes the differences in delivery, and thus mRNA transcript levels, between these two tissues. Therefore combining miRNA repression with other tumor targeting strategies, particularly improved vector tropism or localized delivery, should result in even further repression in the liver (ie. lower mRNA transcript level than hydrodynamic delivery, so greater miRNA repression) with no unwanted repression in tumors (ie. higher mRNA transcript level than hydrodynamic delivery, so still no miRNA repression).

While no one animal model is expected to completely recapitulate the complexity of human HCC there are several rat models of HCC that develop tumors with similar miRNA changes similar to those seen in humans. Kutay and colleagues showed that Fisher 344 rats fed a folic acid, methionine, and choline-deficient (FMD) diet develop HCCs with low levels of miR-122 [9]. A follow-up study in this model also identified down-regulation of miR-34a, miR-127, miR-200b, and miR-16a [49]. Petrelli et al have found that members of the miR-100 family, particularly miR-100 and miR-99a, were down-regulated in both human HCC and in

a chemically-induced model of HCC called the R-H model [50]. Impressively, in our model we discovered that of the twelve miRNAs that we analyzed and were known to be down-regulated in human HCC, seven were also down-regulated in rat HCC, and none of the miRNAs examined were over-expressed. Therefore, our rat HCC model should be of significant interest for those interested in examining the role of miRNA loss in HCC development and testing miRNA therapeutics aimed at correcting miRNA deregulation. Future work will also focus on a more global assessment of the miRNome in this model as new miRNAs down-regulated in human HCC are still being uncovered.

In summary, we have developed miRNA-responsive vectors towards miR-122 and miR-26a and have shown the ability of the corresponding miRNAs to suppress transgene expression in normal liver but have no effect on expression in orthotopic rat HCC. By incorporating miR-26a and/or miR-122 binding sites into future vectors and combining this with other targeting strategies, complete elimination of cytotoxic protein production in hepatocytes should be possible. We believe that continued exploration of this class of vectors will provide greater control over the cell populations that transgene expression is limited to, with the hope to provide vectors that can achieve maximal tumor kill but minimal-to-none normal tissue toxicity. Ultimately, true tumor specificity may be achieved which will lead to an improved safety profile of therapeutic vectors, a greater therapeutic index, and hopefully a cure for this devastating disease.

Materials and Methods

Cell Lines and Media

HUH-7 (human hepatoma) cells were grown in DMEM high glucose medium (Gibco, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS), 1× non-essential amino acids, and 1% Penicillin/Streptomycin (P/S) solution. McA-RH7777 (rat hepatoma) cells were grown in DMEM high glucose medium (Gibco, Carlsbad, CA) supplemented with 10% FBS and 1% P/S solution. HEK-293T (human embryonic kidney cells expressing the large T antigen) cells were grown in MEM (Gibco, Carlsbad, CA) supplemented with 10% FBS and 1% P/S solution.

Orthotopic Rat Hepatocellular Carcinoma Model

Buffalo rats were purchased from Charles Rivers Laboratory (Wilmington, MA). Rats were anesthetized with isoflurane inhalation: for induction, 5% isoflurane was mixed in 1 L O_2/ min; and for maintenance, 2~4 % isoflurane was mixed in 1 L O_2/ min. Analgesia was provided via both a subcutaneous injection of Buprenorphine (0.01–0.05 mg/kg) and an intramuscular injection of Flunixin meglumine (2.5 mg/kg). For tumor implantation, a subxiphoid incision was made and the liver was mobilized to expose the left lateral segment. A total of 10^6 syngeneic McA-RH7777 cells suspended in 100 ml of PBS were injected slowly (typically over 30–60 seconds) under the capsule of the left lateral lobe as previously described [46]. A cotton applicator was applied for 2–3 minutes over the needle insertion site followed by application of ~100 μl of 70% ethanol to the peritoneal cavity to prevent extra-hepatic cell spillage. The incision was then closed in layers with suture. Animal experiments were carried out in accordance with institutional guidelines.

MiRNA Expression Analysis

Total RNA, including miRNA, was extracted from cultured cells (HUH-7, McA-RH7777, and HEK-293T) and liver and tumor samples from rats 2 weeks post-implantation using the mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA). MicroRNA levels were assessed using the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA) and primers specific for the following mature miRNAs: rno-miR-23a; rno-miR-26a; rno-miR-101a; rno-miR-122; rno-miR-125a-5p; rno-miR-125b; rno-miR-130a; rno-miR-139-5p; rno-miR-150; rno-miR-199a-5p; and rno-miR-223. Amplification of U6 snRNA served as an endogenous control to normalize individual miRNA expression data.

Vector Construction

miRNA-regulated vectors were constructed using a recombinant pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA), pCMV-luc2, as a starting backbone (Figure 1B). This construct contains the codon-optimized Firefly luciferase (luc2) gene under a cytomegalovirus (CMV) promoter (pCMV) (Figure 1B). An *XhoI* site immediately after the luc2 stop codon was used to insert the miRNA oligomers shown in Table 1. Five miRNA oligomers, miRT-26a, miRT-122, miRT-139-5p, miRT-150 and miRT-199 were designed to contain 4 tandem miRNA target sites (miRTs) perfectly complementary to the miRNAs miR-26a, miR-122, miR-139-5p, miR-150, and miR-199, respectively. miRNA target sites were separated by linkers between 4 to 6 nucleotides in length. Additionally, control Anti-miRT-26a and Anti-miRT-122 oligomers were designed by replacing the miRTs with their respective anti-sense sequences (Table 1). Four miRT sites were chosen since this provides increased repressive capability compared to 1 or 2 sites, and does not saturate the miRNA so that endogenous miRNA target regulation is not perturbed [21,51]. All plasmids were sequenced to verify the inserted targets. As described below, to normalize for transfection efficiency a pCMV-hRluc expression vector (pcDNA3.1 (+) backbone; Invitrogen, Carlsbad, CA) was used, containing a codon-optimized *Renilla* luciferase (hRluc) gene under a CMV promoter.

In vitro Gene Expression Analysis

For *in vitro* assessment of transgene regulation by miRNAs, 1.5×10^5 HUH-7, 10^5 McA-RH7777, and 3×10^5 293T cells were plated in 12 well plates. Cells were then co-transfected with pCMV-hRluc (10 ng) to assess transfection efficiency and one of the following Fluc–expressing vectors (1.6 μg): pCMV-luc2 (no miRT); pCMV-luc2-miRT-122 (122); pCMV-luc2-miRT-26a (26a); pCMV-luc2-Anti-miRT-122 (Anti-122); or pCMV-luc-Anti-miRT-26a (Anti-26a). Transfections were performed using Lipofectamine 2000 transfection agent (Invitrogen, Carlsbad, CA). Cells were lysed in 1× Passive lysis buffer (Promega, Sunnyvale, CA, USA) on ice and lysate was centrifuged at 14,000 rpm for 5 minutes at 4°C. Supernatant was collected and Fluc and Rluc activity was determined using a Dual Luciferase Assay kit (Promega, Sunnyvale, CA) in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). An integration time of 10 seconds was used for all measurements. The protein content of tissue lysates was determined using a Pierce 660 nm Protein Assay system (Thermo Scientific, Rockford, IL) in a BioTek Synergy 4 microplate reader (BioTek Instruments, Winooski, VT). Fluc luminescence results (RLU) were normalized to both Rluc

activity (RLU) and protein (μg) and data is expressed as a percentage of the luminescence results using the control vector (No miRT).

Mice Hydrodynamic Injections and Bioluminescence Imaging

Vectors were amplified in Top10 *Escherichia coli* (Life Technologies, Grand Island, NY, USA) and purified using Endofree Plasmid Maxi kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Hydrodynamic tail-vein injections (20 μg vector in 2.5 mls PBS over ~5 seconds using a 25G needle) were performed on thirteen female Balb/C mice (20–25 g; Charles River Laboratories, Wilmington, MA) using solution containing PBS alone (mock; n=3), pCMV-luc2 (n=3), pCMV-luc2-Anti-miRT-122 (n=3), or pCMV-luc2-miRT-122 (n=4). 48 hours later bioluminescence imaging was performed. Mice were anesthetized (2–3 % isoflurane mixed with 1 L O_2/ min) and injected intraperitoneally with D-luciferin (3 mg). Ten minutes after injection bioluminescence imaging was performed using a cooled charge coupled device (CCD) camera (Xenogen IVIS Spectrum; Xenogen, Alameda, CA) and photons emitted from transfected livers were collected. Images were analyzed using Xenogen Living Image software (Xenogen, Alameda, CA). Regions of interest were drawn over the liver and average radiance (photons/sec/cm^2/ steradian) was determined.

Rat HCC Tumor Model Hydrodynamic Injection and Luminometer Assay

Vectors were purified using Endofree Plasmid Giga kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Hydrodynamic tail-vein injections (400 μg Fluc vector in PBS (5.25% body weight) over 10–15 seconds using a 23G needle) were performed as previously described [35] on 36 male Buffalo rats two weeks following implantation of McA-RH7777 cells. Rats received one of the following Fluc-expressing vectors: pCMV-luc2 (No miRT; n=11); pCMV-luc2-miRT-122 (122; n=3); pCMV-luc2-miRT-26a (26a; n=3); pCMV-luc2-mIRT-139-5p (139-5p; n=6); pCMV-luc2-miRT-150 (150; n=5), or pCMV-luc2-miRT-199a (199; n=3). pCMV-hRluc (100 μg) was co-injected to normalize for delivery efficiency. Five tumor-bearing rats received mock injections (PBS only).

Two days following hydrodynamic delivery, rats were sacrificed, and tumor and liver tissue was harvested, placed on ice and frozen at −80°C. Tissues were thawed and homogenized in 5 volumes of 1X Passive Lysis buffer (Promega, Sunnyvale, CA) on ice. Lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C and supernatant was collected. Fluc and Rluc activity in both tumor and liver was determined with a Dual Luciferase Assay kit (Promega, Sunnyvale, CA) in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). An integration time of 10 seconds was used for all measurements. The protein content of tissue lysates was determined using a Pierce 660 nm Protein Assay system (Thermo Scientific, Rockford, IL) in a BioTek Synergy 4 microplate reader (BioTek Instruments, Winooski, VT). Fluc luminescence results (RLU) were normalized to both Rluc activity (RLU) and protein (μg) and data is expressed as a percentage of the luminescence results using the control vector (No miRT).

Statistics

Data was analyzed using two-tailed t-test when comparing data between two groups or one-way ANOVA followed by Tukey's post-hoc test if comparing 3 groups or more. Nominal p-values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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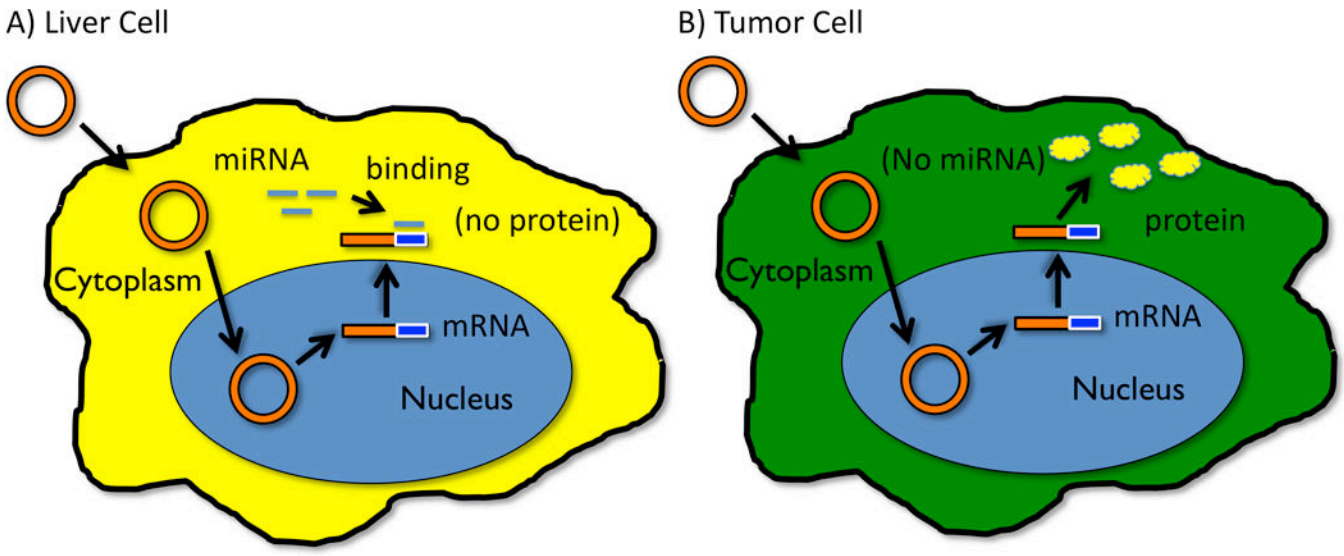

Figure 1.

Improving the tumor specificity of gene therapy vectors through differential miRNA-regulation of transgene expression in liver and tumor cells. A) In liver cells, efficient liver de-targeting is achieved since particular miRNAs are expressed at high levels and bind to their corresponding miRNA binding sites on mRNA transcripts expressed from engineered vectors. B) In tumor cells, these same miRNAs are expressed at significantly lower levels (or absent) and therefore transgene expression continues unhindered.

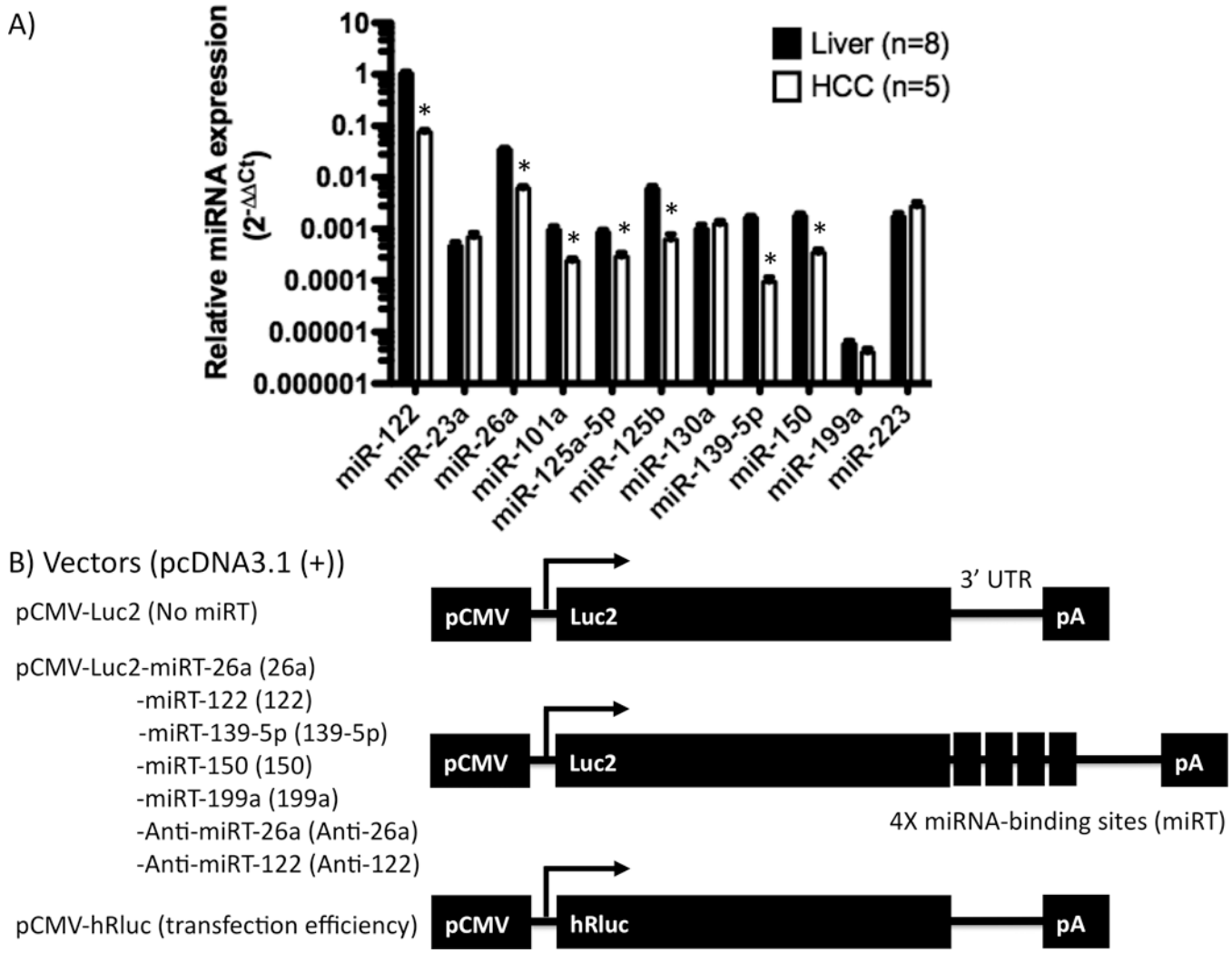

Figure 2.

A) miRNA expression levels in both rat liver and tumor 2 weeks after McA-RH7777 cell implantation into the rat liver. Asterisk represents significant difference at p-value <0.05. Error bars represent mean + SEM. B) Vector diagrams of control vector (No miRT) expressing Firefly luciferase (Luc2) driven by the CMV promoter (pCMV) and miRNA-regulated vectors with 4 tandem sense or anti-sense (Anti-) miRNA-binding sites (miRTs) to a specific miRNA inserted into the 3' untranslated region (3' UTR) of the control vector. To control for transfection efficiency we also constructed a pCMV-driven vector expressing humanized renilla luciferase (hRluc).

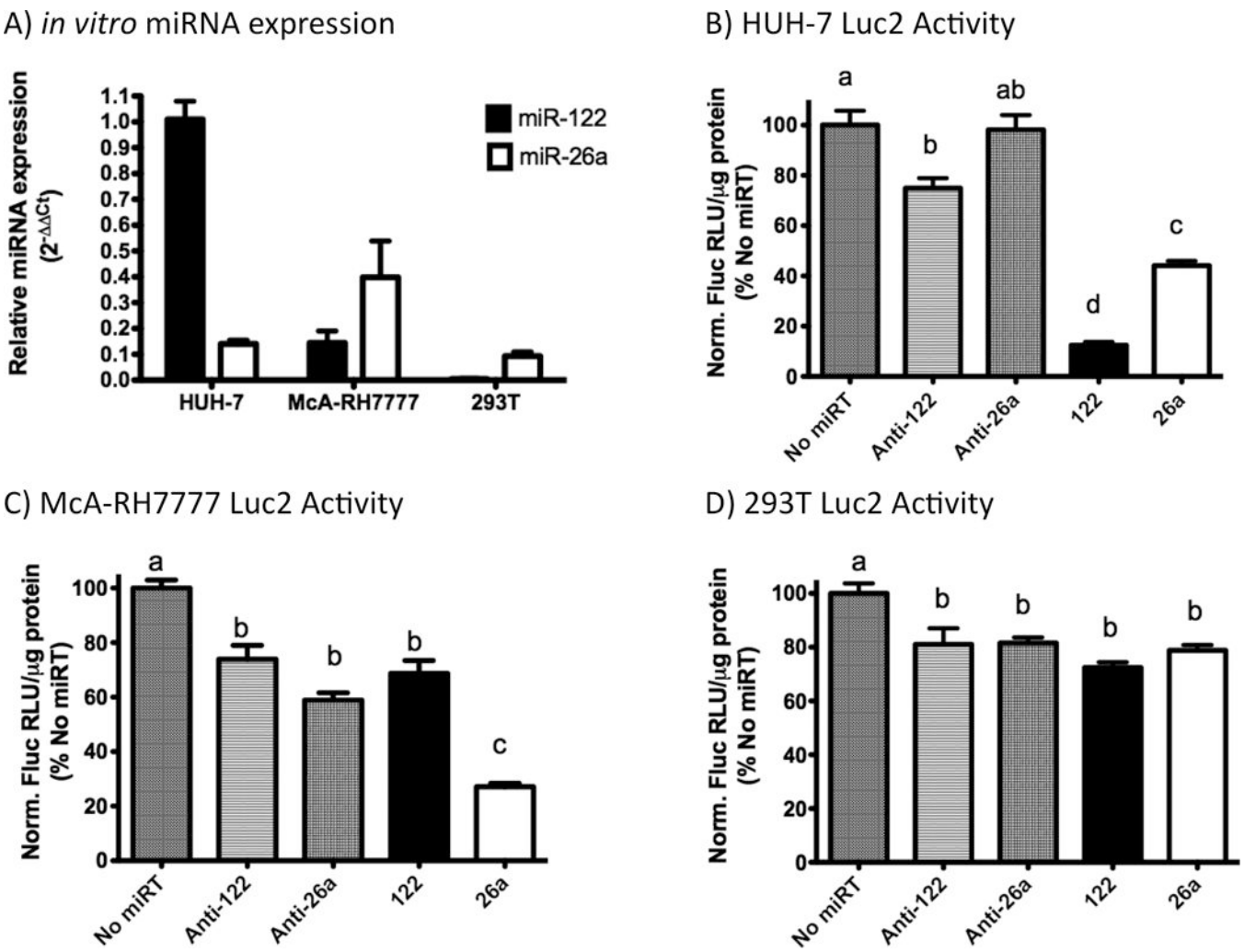

Figure 3.

A) Relative expression levels of miR-122 and miR-26a in HUH-7, McA-RH7777, and 293T cells. Data is normalized to expression levels of miR-122 in HUH-7 cells. B–D) Normalized Fluc activity after transient transfection with control (No miRT, Anti-122, and Anti-26a) or miRNA-regulated (122 and 26a) vectors in HUH-7, McA-RH7777, and 293T cells, respectively. Bars with different letters above them represent a significant difference at a p-value <0.05. Experiments were performed in triplicate and error bars represent mean + SEM.

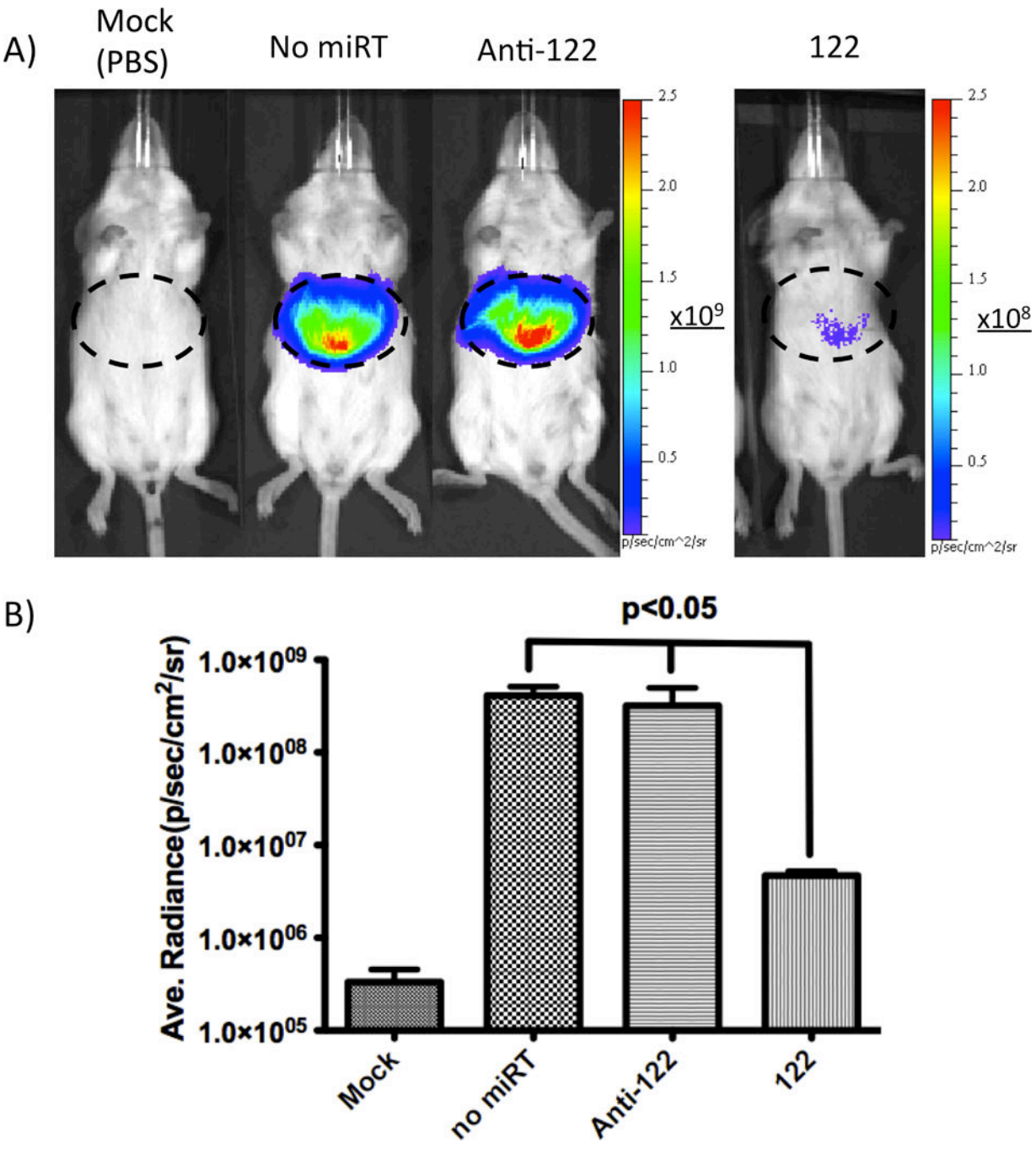

Figure 4.

A) In vivo bioluminescence imaging of non-tumor bearing Balb/C mice after hydrodynamic delivery of PBS alone (Mock), control (No miRT and Anti-122) vectors, or a liver-specific miRNA-regulated (122) vector. A region-of-interest (ROI) was drawn over the liver region of each mouse to quantify bioluminescence signal. B) Quantitative analysis of bioluminescence signal from liver using each vector. Approximately two orders of magnitude lower signal was seen with the 122 vector versus the control vectors ($p<0.05$). Error bars represent mean + SEM of 3 mice.

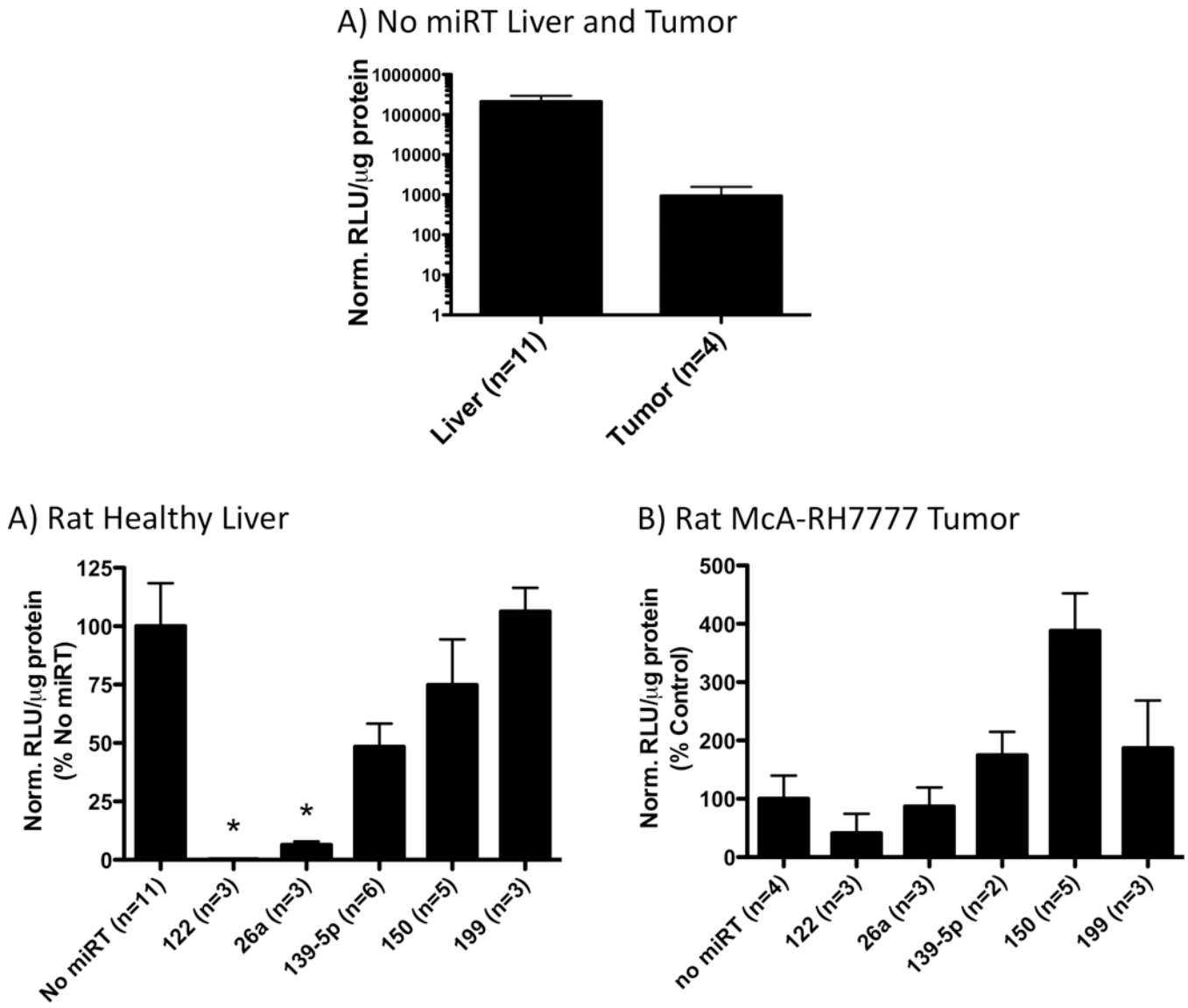

Figure 5.

A) Absolute normalized Fluc activity of the No miRT vectors within the liver and tumor. B–C) Normalized Fluc activity within the liver (B) and tumor (C) of McA-RH7777 tumor-bearing rats following hydrodynamic delivery of control (No miRT) and miRNA-regulated (122, 26a, 139-5p, 150, 199) vectors. Data is expressed as a percentage compared to No miRT vector. Asterisk represents significant difference versus No miRT vector at a p-value of <0.05. Error bars represent mean + SEM.

Table 1

microRNA oligomers used in this study

miRNA oligomers	Sequence
miRT-26a	5’*ctcgag*CTCTAGA**agcctatcctggattacttgaa**CGAT**agcctatcctggattacttgaa**ACCGGT**agcctatcctggattacttgaa**TCAC**agcctatcctggattacttgaa**GGATCC*ctcgag*3’
miRT-122	5’*ctcgag*CTCTAGA**caaacaccattgtcacactcca**CGAT**caaacaccattgtcacactcca**ACCGGT**caaacaccattgtcacactcca**TCAC**caaacaccattgtcacactcca**GGATCC*ctcgag*3’
miRT-139-5p	5’*ctcgag*CTCTAGA**ctggagacacgtgcactgtaga**CGAT**ctggagacacgtgcactgtaga**ACCGGT**ctggagacacgtgcactgtaga**TCAC**ctggagacacgtgcactgtaga**GGATCC*ctcgag*3’
miRT-150	5’*ctcgag*CTCTAGA**cactggtacaagggttgggaga**CGAT**cactggtacaagggttgggaga**ACCGGT**cactggtacaagggttgggaga**TCAC**cactggtacaagggttgggaga**GGATCC*ctcgag*3’
miRT-199a	5’*ctcgag*CTCTAGA**gaacaggtagtctgaacactggg**CGAT**gaacaggtagtctgaacactggg**ACCGGT**gaacaggtagtctgaacactggg**TCA**C**gaacaggtagtctgaacactggg**GGATCC*ctcgag*3’
Anti-miRT-26a	5’*ctcgag*CTCTAGAt**cggataggacctaatgaactt**CGAT**tcggataggacctaatgaactt**ACCGGT**tcggataggacctaatgaactt**TCAC**tcggataggacctaatgaactt**GGATCC*ctcgag*3’
Anti-miRT-122	5’*ctcgag*CTCTAGA**gtttgtggtaacagtgtgaggt**CGAT**gtttgtggtaacagtgtgaggt**ACCGGT**gtttgtggtaacagtgtgaggt**TCAC**gtttgtggtaacagtgtgaggt**GGATCC*ctcgag*3’