INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third 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. Whereas surgical resection and liver transplantation are the best curative options, only a small proportion (~20%) of patients are candidates for such procedures. Therefore, new therapies are urgently needed to stem the overwhelming mortality associated with this deadly disease.

MicroRNAs (miRNAs) are a large class of highly conserved, short (~22 nucleotides), noncoding RNAs that bind to imperfectly complementary sequences in the 3’ untranslated regions of target mRNAs.2 In many species including humans, upon binding to miRNAs, 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.3,4 In humans, ~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 deregulated 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 in 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. As miRNA expression levels in cancer cells compared with 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 downregulated in HCC, and explore the ability of these miRNA-regulated vectors to improve the tumor specificity of transgene expression in living subjects.

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RESULTS

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

Our first goal was to identify miRNAs that had lower expression in rat and human HCC versus normal liver. Rather than performing a broad search (for example, 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 miRNA-regulated strategy should have the highest translational potential. The following 11 miRNAs were identified as potential targets: miR-23a;3,25 miR-27a;12,11,16 miR-101a;2,9–11,30 miR-122;1,3,12,31,32 miR-125a-5p;31 miR-125b;31 miR-130a;31 miR-133a;20 miR-140;2,3 miR-141;1 miR-143;2,3 miR-150;32 miR-199a-5p12,32 and miR-223.32 Expression plasmids expressing the firefly luciferase (Luc2) gene driven by a cytomegalovirus (CMV) promoter (pCMV) to carry four tandem miRNA-regulated vectors with four tandem sense or antisense (Anti-) target 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).

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 cytomegalovirus (CMV) promoter (pCMV) to carry four tandem sense or antisense (Anti-) target sites (miRTs) to the following miRNAs within the 3’-untranslated region: miRT-26a (26a); miRT-122 (122); miRT-129-5p (129-5p); miRT-150 (150); miRT-199a (199a), Anti-miRT-26a (Anti-26a) and 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, as 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’-untranslated region by inclusion of antisense miRNA-binding sites significantly decreased Fluc activity by an overall average of 22.3 ± 15.0% across all cell lines (P < 0.05) (Figures 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 antisense 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. This vector was chosen, as miR-122 accounts for ~70% of all miRNAs expressed in the liver. Forty-eight hours after vector delivery, bioluminescence imaging was performed and regions of interest over the liver were analyzed for Fluc expression. 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 with the No miRT vector. Importantly, the 122 vector showed almost two orders of magnitude lower Fluc activity compared with both other control vectors (1.15% versus No miRT; $P < 0.05$).

Differentially expressed miRNAs 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, as 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 $\sim 200 – 1000 \text{mm}^3$ in size develops over the course of 2 weeks post-cell implantation (ultrasound measurements; JA Ronald, R Katzenberg, LV Hofmann, 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, as miR-199 was found to be expressed at extremely low levels in both liver and tumor (Figure 2a). Each Fluc vector (400 $\mu$g) was hydrodynamically injected into tumor-bearing rats along with our Rluc vector (100 $\mu$g) to control for delivery. Both liver and tumor were harvested 48 h 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 phosphate-buffered saline (PBS) to determine the background level of Rluc activity and identify tissues that were transfected and further analyzed (that is, 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, as 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 $\sim 100$-fold higher in liver than in 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 the liver or tumor (Figures 5b and c). In both liver and tumor, no difference in Fluc activity was detectable between the No miRT and 199 control vectors (Figures 5b and c). Importantly, within the liver both the 122 and 26a vectors had significantly lower Fluc expression ($P < 0.05$) compared with both control vectors (Figure 5b). In striking contrast, these same vectors did not result in significant repression of Fluc activity within the tumor (although the 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 the cancer cells so that unwanted toxicity in normal tissues is prevented. In most HCC patients, normal liver health is already compromised, therefore 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, as 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 two miRNAs, miR-122 and miR-26a, that are downregulated in both our rat model of HCC and human HCC, and have shown that miRNA-sensitive vectors towards these two 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. Jin et al. recently showed that incorporation of eight 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. 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 with normal liver. Therefore, in patients harboring Let-7-positive HCC, theoretically this Let-7-sensitive vector would not be efficacious, as 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 downregulation is not a rule in HCC.\textsuperscript{5,16} Owing to this heterogeneity in miRNA expression across HCC tumors with varying etiology, one can imagine the development of a suite of ‘personalized’ therapeutic vectors regulated by one or more miRNAs (miR-122, miR-26a, Let-7 and so on) that would be administered to individual patients based on the miRNA transcriptome 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.\textsuperscript{10}

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,\textsuperscript{41} localized intratumoral\textsuperscript{42,43} or intra-arterial administration,\textsuperscript{44} and tissue-specific regulation of transgene expression.\textsuperscript{43,45,46} Fu et al.\textsuperscript{28} recently pursued this latter type of strategy by combining the use of the liver-specific apolipoprotein E-AAT promoter with miR-122a regulation to restrict replication of an oncolytic herpes simplex virus in HCC xenografts after intratumoral injection. 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 with vectors driven by the strong, constitutive CMV promoter.\textsuperscript{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. 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. 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, and therefore 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, as increasing the length of the 3′-untranslated region may have unwanted effects of expression levels.

As highlighted recently, the degree of miRNA repression is dictated by both miRNA and mRNA transcript levels, 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. We confirmed these results because in many primarily delivers constructs to the liver, with minimal delivery by the delivery method. Hydrodynamic delivery of vectors driven by strong promoters will be dictated predominantly the amount of miRNA repression in both liver and tumor of (our TSTA system). Therefore, incorporating miRNA-binding sites expression within the liver, which was most likely amplified by promoters, our vector did show some background ‘leaky’ miRTs, as increasing the length of the 3′-untranslated region may have unwanted effects of expression levels.

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 the 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 the 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
isoflurane was mixed in 1 l O2 min

46 A cotton applicator was applied for 2–3 min over the needle to expose the left lateral segment. A total of 106 syngeneic McA-RH7777 cells were injected into the peritoneal cavity to prevent extrahepatic cell spillage. The incision was closed with 4–0 surgical thread. The animals were monitored daily for 2 weeks post implantation using the mirVana miRNA Isolation kit (Applied Biosystems). MicroRNA expression data were analyzed using the program Genes (Thermo Scientific, Rockford, IL, USA) in a BioTek Synergy 4 microplate reader (BioTek Instruments, Winooski, VT, USA). Fluc luminescence results (RLU) were normalized to both Rluc activity (RLU) and protein content of tissue lysates determined using a Pierce 660 nm Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

**MATERIALS AND METHODS**

**Cell lines and media**

HUH-7 (human hepatoma) cells were grown in DMEM high-glucose medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1 x nonessential amino acids and 1% Penicillin/Streptomycin solution. McA-RH7777 (rat hepatoma) Cells were grown in DMEM high-glucose medium (Gibco) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin solution. HEK-293T (human embryonic kidney cells expressing the large T antigen) cells were grown in MEM (Gibco) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin solution.

**Orthotopic rat HCC model**

Buffalo rats were purchased from Charles Rivers Laboratory (Wilmington, MA, USA). Rats were anesthetized with isoflurane inhalation: for induction, 5% isoflurane was mixed in 11 O2 min−1; and for maintenance, 2–4% isoflurane was mixed in 11 O2 min−1. Analgesia was provided via both a subcutaneous injection of Buprenorphine (0.01–0.05 mg kg−1) and an intramuscular injection of Flunixin meglumine (2.5 mg kg−1). For tumor implantation, a subxiphoid incision was made and the liver was mobilized to expose the left lateral segment. A total of 105 syngeneic McA-RH7777 cells suspended in 100 ml of PBS were injected slowly (typically over 30–60 s) under the capsule of the left lateral lobe, as previously described.46 A cotton applicator was applied for 2–3 min over the needle insertion site followed by the application of ~ 100 μl of 70% ethanol to the peritoneal cavity to prevent extraperitoneal 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, USA). miRNA levels were assessed using the TaqMan MicroRNA assay (Applied Biosystems) and primers specific for the following mature miRNAs: no-miR-23a; no-miR-26a; no-miR-101a; no-miR-122; no-miR-125a-5p; no-miR-125b; no-miR-130a; no-miR-139-5p; no-miR-150; no-miR-199a-5p and no-miR-223. Amplification of U6 small nuclear RNA 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, USA), pCMV-Luc2, as a starting backbone (Figure 1b). This construct contains the codon-optimized firefly luciferase (Luc2) gene under a CMV promoter (pCMV) (Figure 1b). An Xho I 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-199a, were designed to contain four 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 four and six nucleotides in length. Additionally, control Anti-miRT-26a and Anti-miRT-122 oligomers were designed by replacing the miRTs with their respective antimir sequences (Table 1). Four miRT sites were chosen, as this provides increased repressive capability compared with one or two 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) was used, containing a codon-optimized Renilla luciferase (hrLuc) gene under a CMV promoter.

**In vitro gene expression analysis**

For in vitro assay of transgene regulation by miRNAs, 1.5 × 105 HUH-7, 1.0 × 105 MCA-RH7777 and 3 × 105 293T cells were plated in 12-well plates. Cells were then cotransfected 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-Luc2-Anti-miRT-26a (Anti-26a). Transfections were performed using Lipofectamine 2000 transfection agent (Invitrogen). Cells were lysed in 1 × passive lysis buffer (Promega, Sunnyvale, CA, USA) on ice and lysate was centrifuged at 14 000 r.p.m. for 5 min at 4 °C. Supernatant was collected, and Fluc and Rluc activity were determined using a Dual Luciferase Assay Kit (Promega) in a TD 20/20 luminescence reader (Turner Diagnostics, Sunnyvale, CA, USA). An integration time of 10 s was used for all measurements. The protein content of tissue lysates was determined using a Pierce 660 nm Protein Assay kit (Thermo Scientific, Rockford, IL, USA) in a BioTek Synergy 4 microplate reader (BioTek Instruments, Winooski, VT, USA). Fluc luminescence results (RLU) were normalized to both Fluc activity (RLU) and protein (μg), and data are 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, USA) according to the manufacturer’s instructions. Hydrodynamic tail-vein injections (20 μg vector in 2.5 ml PBS over ~5 s using a 25G needle) were performed on 13 female Balb/c mice (20–25 g; Charles River Laboratories) 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). After 48 h, bioluminescence imaging was performed. Mice were anesthetized (2–3% isoflurane mixed with 11 O2 min−1) and

| miRT oligomers | Sequence |
|----------------|----------|
| miRT-26a | 5'-ctcgagCTCTAGAagccatctctgtgactttgaaCTACTatactcttctgaaggATCGCctcgag-3' |
| miRT-122 | 5'-ctcgagCTCTAGAaacaacactcttgacactcaacGAGTGACTATgtgatcactcaacCAATGCGCctcgag-3' |
| miRT-139-5p | 5'-ctcgagCTCTAGAagtgaacacgtctgtgactttgaaCTACTatactcttctgaaggATCGCctcgag-3' |
| miRT-150 | 5'-ctcgagCTCTAGAaacaacactcttgacactcaacGAGTGACTATgtgatcactcaacCAATGCGCctcgag-3' |
| miRT-199a | 5'-ctcgagCTCTAGAagtgaacacgtctgtgactttgaaCTACTatactcttctgaaggATCGCctcgag-3' |
| Anti-miRT-26a | 5'-ctcgagCTCTAGAagtgaacacgtctgtgactttgaaCTACTatactcttctgaaggATCGCctcgag-3' |
| Anti-miRT-122 | 5'-ctcgagCTCTAGAagtgaacacgtctgtgactttgaaCTACTatactcttctgaaggATCGCctcgag-3' |

Italicized nucleotides are restriction sites and bold nucleotides are sense or anti-sense microRNA target sites (miRTs).

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injected intraperitoneally with d-luciferin (3 mg). Ten minutes after injection, bioluminescence imaging was performed using a cooled charge coupled device camera (Xenogen IVIS Spectrum; Xenogen, Alameda, CA, USA) and photons emitted from transfected livers were collected. Images were analyzed using Xenogen Living Image software (Xenogen). Regions of interest were drawn over the liver and average radiance (photons s⁻¹ cm⁻² sr⁻¹) was determined.

Rat HCC tumor model hydrodynamic injection and luminometer assay

Vectors were purified using Endofree Plasmid Giga kits (Qiagen) according to the manufacturer’s instructions. Hydrodynamic tail-vein injections (400 μg Fluc vector in PBS (5.25% body weight) over 10–15 s using a 23G needle) were performed, as previously described, 25 on 36 male Buffalo rats 2 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-26a (139-Sp; n = 6); pCMV-Luc2-miRT-150 (150; n = 5) or pCMV-Luc2-miRT-199a (199; n = 3). pCMV-RLuc (100 μg) was coinjected to normalize for delivery efficiency. Five tumor-bearing rats received mock injections (PBS only).

Two days following hydrodynamic delivery, rats were killed, and tumor and liver tissue was harvested, placed on ice and frozen at −80 °C. Tissues were thawed and homogenized in five volumes of 1 × Passive Lysis buffer (Promega) on ice. Lysates were centrifuged at 14 000 rpm for 15 min at 4 °C and supernatant was collected. Fluc and Rluc activity in both tumor and liver homogenates was measured using a luminometer (Xenogen). Data were analyzed using two-tailed t-test when comparing data between two groups or one-way ANOVA, followed by Tukey’s post hoc test when comparing three groups or more. Nominal P-values <0.05 were considered significant.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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