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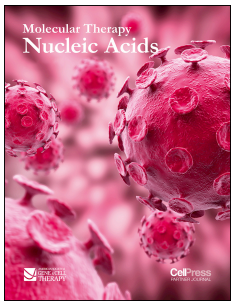

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Bijay Dhungel, Charmaine A. Ramlogan-Steel, Christopher J. Layton, Jason C. Steel

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microRNA199a based post-transcriptional detargeting of gene vectors for Hepatocellular Carcinoma

Bijay Dhungel[1, 2, 3], Charmaine A. Ramlogan-Steel[2], Christopher J. Layton [2], Jason C. Steel[2]

[1]Gallipoli Medical Research Institute, Greenslopes Private Hospital, 102 Newdegate Street, Brisbane, QLD 4120, QLD, AUS

[2]Faculty of Medicine, The University of Queensland, 288 Herston Road, Herston, Brisbane, QLD 4006, QLD, AUS

[3]University of Queensland Diamantina Institute, Translational Research Institute, 37 Kent Street, Woolloongabba QLD 4102, QLD, AUS

Corresponding Author:

Jason C Steel

Faculty of Medicine, The University of Queensland, 288 Herston Road, Herston, Brisbane, QLD 4006, QLD, AUS

E-mail: j.steel2@uq.edu.au

Phone +61 7 33460611

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Abstract

A gene therapeutic platform needs to be both efficient and safe. The criterion of safety is particularly important for diseases like hepatocellular carcinoma (HCC) which develop in a background of an already compromised liver. Gene vectors can be constructed either by targeting HCC or by detargeting liver and/or other major organs. miRNA based negative detargeting has gained considerable attention in recent times due to its effectiveness and the ease with which it can be adapted into current gene delivery vectors. In this study, we provide a proof-of-concept of using miRNA199a as a negative targeting agent. We introduced vectors harbouring reporters with miRNA199a binding sites in cells expressing high endogenous levels of miRNA199a and compared the reporter expression in HCC cells with low endogenous miRNA199a. We observed that the expression of reporters with miRNA199a binding sites is significantly inhibited in miRNA199a positive cells whereas minimal effect was observed in miRNA199a negative HCC cells. In addition, we created a post-transcriptionally regulated suicide gene therapeutic system based on cytosine deaminase (CD)/ 5-fluoro cytosine (5-FC) exploiting miRNA199a binding sites and observed significantly lower cell death for miRNA199a positive cells. Furthermore, we observed a decrease in levels of miRNA199 in 3D tumourspheres of miRNA199a positive Hepa1-6 cells and a reduction in the inhibition of reporter expression after transfection in these 3D models when compared with 2D Hepa1-6 cells. In summary, we provide evidences of miRNA199a based post-transcriptional detargeting with relevance to HCC gene therapy.

Introduction

Hepatocellular carcinoma (HCC) accounts for the majority of liver cancer, one of the cancer types with high incidence-to-mortality rates [1]. The limitation of available therapies, especially at advanced stages, is clearly highlighted by their palliative nature and rising trends in disease-associated mortality and morbidity rates [1, 2]. Moreover, the presence of underlying liver dysfunction in most patients limit the application of conventional therapies like radio and chemotherapy [3]. Sorafenib, with only a modest survival benefit, is the only FDA approved drug for late stage HCC [4]. Given the aetiology of HCC, targeted therapies which can limit the treatment to HCC while sparing the liver could have a significant therapeutic benefit [5]. In this regard, gene therapy, which provides several strategies that can be exploited to target a certain cell type, may be particularly attractive [5, 6].

Gene therapy offers different approaches for cancer targeted gene delivery, these include (but are not limited to) modification of gene delivery vehicle (vector) and modification of the therapeutic payload (controlled expression of the transgene) [7]. The former mostly comprises of the use of capsid modified viral vectors with an altered tropism, displaying preference for cancer cells [6]. Limiting the expression of the therapeutic gene in cancer cells by transcriptional targeting i.e. exploiting tumor specific promoters is another strategy with proven efficacy [8, 9], however, this method is limited by the number of available promoters with a strong HCC specificity [6, 10]. Post-transcriptional regulation of gene expression by utilizing cell-specific endogenous microRNAs (miRNAs) is an emerging approach for targeted gene expression. Since the first evidence of effective application of this approach in antigen presenting cells [11], a number of studies have successfully used binding sites of tissue, or disease specific miRNAs for regulating transgene expression [12-14]. In this negative targeting method, binding sites of miRNA expressed at high levels in target cells is

incorporated at the UTRs of transgene and as a result transgene expression gets inhibited in those cells (Figure 2a) [15].

In this proof-of-concept study, we demonstrate miRNA199a based detargeting after gene delivery. First, we probed the expression of miRNA199a in cryopreserved human hepatocytes, HepaRG and a panel of HCC and non-HCC cell lines. After observing a significant downregulation of miRNA199a in HCC and non-HCC tumor cell lines (except for Hepa1-6 which expressed miRNA199a at levels comparable to primary hepatocytes and HepaRG cells), we constructed expression vectors with miRNA199a binding sites at the 3'-UTR of reporters eGFP and gaussia luciferase (GLuc) and transfected both miRNA199a positive and negative cells with these plasmids. A significant inhibition of the expression of reporter was observed in cells with high endogenous miRNA199a levels while negligible effects were seen in others. These findings were further validated by targeted gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase/5-fluorocytosine (CD/5-FC) system. Next, we demonstrate that the inhibition of miRNA199 in miRNA199 positive cells can rescue the expression of transgenes with miR199a binding sites while its overexpression with miRNA mimic can inhibit the same in miRNA199a negative HCC cells.

Furthermore, the possibility of delivering these constructs with Adeno associated virus (AAV) based delivery system was explored and the results obtained with transfection experiments were corroborated. Additionally, we discovered that the levels of miRNA199a is downregulated in $CD133^+$ $CD44^+$ $Oct4^+$ expressing tumorspheres of Hepa1-6 illustrating the potential to use miRNA199a to target these 3D models of HCC. Finally, we demonstrate that tumorspheres of Hepa1-6 can be efficiently targeted with miRNA199a binding site containing expression vectors. In conclusion, this study provides evidence that negative targeting after gene delivery can be achieved in hepatocytes by exploiting miRNA199a and

suggests that this principle of negative targeting with miRNA199a could be exploited for other cell types expressing high endogenous miRNA199a levels.

Results

miRNA199a is downregulated in HCC and other cancer cell lines

To investigate the expression pattern of miRNA199a, we performed real time qPCR on cDNA obtained from total RNA extracted from primary hepatocytes, HepaRG and a panel of HCC and non-HCC cell lines. After quantifying the amounts of miRNA199a with qPCR, high levels of miRNA199a was observed in cryopreserved human primary hepatocytes and HepaRG cells (92 copies per 1000 copies of control) **(Figure 1a)**. Similarly, the HCC cell line Hepa1-6 was found to have higher copies of miRNA199a (498 copies per 1000 copies of RNU control) **(Figure 1a)**. In HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423, no copies of miRNA199a was detected using this method. Similarly, little or no expression of miRNA199a was observed in the non-HCC cancer cell lines used in this study **(Figure 1b)**. Together, these results indicated that miRNA199a is downregulated in HCC as well as non-HCC tumor cell lines and established Hepa1-6 as a model to study different aspects of miRNA199a.

Incorporation of miRNA199a binding sites at the 3'-UTR of a transgene allows negative targeting of cells with high endogenous expression of miRNA199a

To explore the effects of inclusion of miRNA199a binding sites at the 3'-UTR of a transgene, we constructed expression plasmids with GLuc with 3 miRNA199a binding sites at the 3'-UTR (CMV-GLuc-miR199a*3). These plasmids were then transfected into miRNA199a positive cell lines HepaRG, and Hepa1-6 as well as miRNA199a negative HCC, and non-HCC cell lines. The percentage of secreted luciferase after transfection with CMV-GLuc-miR199a*3 was observed to be significantly lower when compared to that after transfection

with CMV-GLuc for both HepaRG ($p<0.005$), and Hepa1-6 ($p<0.001$) (**Figure 2b**). Interestingly, the decrease in luciferase expression after transfection with CMV-GLuc-miR199a*3 in HepaRG cells was observed to be 2.94 times ($p<0.001$) higher than that observed for Hepa1-6 which correlated with the copies of miRNA199a present in those cells. As expected no significant decrease in GLuc expression was observed in HCC cell lines Hep3B, SKHep1, PLC/PRF/5, and SNU423 (**Figure 2c**) as well as non-HCC tumor cell lines (**Figure 2d**) after transfection with CMV-GLuc-miR199a*3. To further confirm this observed miRNA199a mediated post transcriptional control of gene expression, we used a second reporter eGFP. Hepa1-6 cells were transfected with either CMV-eGFP or CMV-eGFP-miR199a*3 and GFP positive cells were quantified by flow cytometry. Like the GLuc reporter, a significantly reduced GFP expression was observed after transfection with CMV-eGFP-miR199a*3 when compared to CMV-eGFP ($p<0.001$) (**Figure 2e**). Together, these results provided evidence that the incorporation of miRNA199a binding sites at the 3'-UTR of a gene can inhibit its expression in cells with high endogenous levels of miRNA199a.

Overexpression of miRNA199a in HCC cells inhibits expression of transgene with its binding sites at the 3'-UTR while its inhibition rescues the expression of the same in cells with high endogenous expression levels

Next, we altered miRNA199a levels in Hepa1-6 and HCC cells using inhibitors and mimics to define miRNA199a's role in the expression of reporters with binding sites incorporated in the 3'-UTR. We co-transfected miRNA199a inhibitor with either CMV-eGFP-199a*3 or CMV-GLuc-miR199a*3 and compared expression to CMV-GLuc, or CMV-eGFP controls. A significant increase ($p<0.05$) in the percentage of GFP positive cells was observed after co-transfection of Hepa1-6 with CMV-eGFP-miR199a*3 and miRNA199a inhibitor when compared to transfection with CMV-eGFP-miR199a*3 (both reported as relative to CMV-eGFP) (**Figure 3a, b**). Similarly, miRNA199a inhibition by co-transfection of Hepa1-6 with

CMV-GLuc-miR199a*3 and miRNA199a inhibitor resulted in a 2.9-fold increase ($p<0.05$) of GLuc expression when compared to GLuc expression after transfection with CMV-GLuc-miR199a*3 alone (both normalized to CMV-GLuc control) (**Figure 3c**). In contrast, co-transfection of CMV-GLuc-miR199a*3 and miRNA199a mimic in miRNA199a negative HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423 resulted in a significant decrease (**Figure 3d**) ($p<0.05$) in GLuc expression when compared to CMV-GLuc-miR199a alone (all normalized to CMV-GLuc control). Taken together, these results further strengthen the observation of miRNA199a mediated post-transcriptional regulation of expression of reporter gene containing its binding sites at the 3'-UTR and provide evidence that its inhibition can rescue the expression of these reporters.

Post-transcriptionally controlled gene directed enzyme prodrug therapy (GDEPT) for HCC

Next, we constructed a post-transcriptionally regulated GDEPT platform based on suicide gene CD and prodrug 5-FC for gene therapy. Either CMV-CD or CMV-CD-miR199a*3 were transfected into miRNA199a positive Hepa1-6 as well as miRNA199a negative HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423. We studied both cell proliferation and cell death (**Figure 4**) after transfection with CMV-CD, and CMV-CD-miR199a*3. A 1.8-fold higher proliferation rate was observed for Hepa1-6 after transfection with CMV-CD-miR199a*3 when compared to transfection with CMV-CD ($p<0.05$) and incubation with prodrug 5-FC whereas no significant difference was observed in HCC cell lines Hep3B, PLC/PRF/7, SKHep1, and SNU423 (**Figure 4a**). Similarly, total cell death (quantified by PI/annexin staining) was significantly lower ($p<0.001$) in Hepa1-6 after transfection with CMV-CD-miR199a*3 and incubation with 5-FC when compared to CMV-CD positive control (**Figure 4b, c**) while no significant effects were observed in HCC cell lines Hep3B, PLC/PRF/7, and SKHep1 (**Figure 4b, d**). These results demonstrate that a liver detargeted

GDEPT system which simultaneously targets HCC can be constructed using miRNA199a mediated post-transcriptional gene regulation.

Adeno associated virus (AAV) mediated delivery of transgenes with miR199a binding site for detargeting cells containing high endogenous miRNA199a levels

To examine the possibility of delivering miRNA199a based post-transcriptionally regulated reporter and therapeutic genes using AAV vectors, we constructed self-complementary AAV serotype 8 with or without miR199a binding sites at the 3'-UTR of the transgenes (scAAV8-GLuc, scAAV8-GLuc-miR199a*3, scAAV8-CD, scAAV8-CD-miR199a*3). Transduction of miRNA199a positive Hepa1-6 cell line with scAAV8-GLuc-miR199a*3 resulted in a significantly reduced GLuc expression ($p<0.05$) when compared to scAAV8-GLuc while no significance difference in GLuc expression was observed in miRNA199a negative HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423 (**Figure 5b**). Similarly, after transduction with scAAV8-CD-miR199a*3 and subsequent incubation with prodrug 5-FC, a 3.4-fold higher proliferation rate was observed in Hepa1-6 cells ($p<0.05$) when compared to scAAV8-CD whereas no significant difference in cell proliferation was observed for both groups in HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423 (**Figure 5c**). These results demonstrate the compatibility of our miRNA199a based post-transcriptionally targeted gene delivery and therapy system with AAV vector based delivery system, the most widely used delivery vector for therapeutic purposes.

$CD133^+$ $CD44^+$ $Oct4^+$ enriched 3D tumorspheres can be targeted with vectors harbouring transgenes with miRNA199a binding sites at the 3'-UTR

In order to assess the efficacy of miRNA199a targeting in a more complex tumor-like environment, we utilized Hepa1-6 cells grown under 3D tumorsphere culture conditions (**Figure 6a**). These heterogeneous 3D tumor-like cultures have also been shown to enrich for

cancer stem cells [16]. Given the reported tumor supressing roles of miRNA199a and its downregulation in cancer stem cells for other cancer types, we first investigated whether maintaining Hepa1-6 cells as 3D tumorspheres could enrich for classic cancer stem cell markers. We quantified the levels of expression of HCC stemness markers CD44 (**Figure 6b**), CD133 (**Figure 6c**), and Oct4 (**Figure 6d)** in these tumorspheres and observed a significant upregulation of these genes when compared to 2D grown Hepa1-6 cells ($p<0.05$). The increase in stemness markers correlated to a downregulation in levels of miRNA199a (**Figure 6e**).

Next, we examined whether Hepa1-6 cells tumor-like spheroids could be efficiently targeted by the miR199a TS vectors. To do this, we transfected these stem-cell enriched 3D tumorspheres of Hepa1-6 and 2D Hepa1-6 with CMV-GLuc-miR199a*3 and examined reported expression. We observed a significant increase in the reporter expression in 3D tumorspheres compared to the miRNA199a expressing 2D cultures ($p<0.05$) (**Figure 6f**). Similarly, a 2.19-fold increase in GLuc expression ($p<0.005$) was observed after transduction of 3D tumorspheres of Hepa1-6 with scAAV8-GLuc-miR199a*3 when compared to 2D culture (**Figure 6g**). Using GDEPT with CD/5-FC system also resulted in a significantly higher cell death ($p<0.001$) in 3D tumorspheres of Hepa1-6 cells when compared to the 2D culture (**Figure 6h**). These results suggest that miRNA199a targeting strategies have utility in targeting more complex tumor-like spheroids and may also have utility in targeting hepatocellular CSC with decreased miRNA199 expression.

Discussions

miRNAs are small, untranslated, endogenous RNA molecules that efficiently regulate the expression of a gene by binding to a specific sequence in its mRNA (binding sites). In addition to having a cell specific expression pattern and being involved in several important

biological processes ranging from development to apoptosis, several miRNAs have been reported to be dysregulated in several diseases including cancer. Inclusion of binding sites of miRNAs that are downregulated in cancer while being expressed at high levels in normal cells is an attractive approach for limiting transgene expression in cancer cells.

In this study we first, investigated the expression levels of miRNA199a in hepatocytes and a panel of HCC and non-HCC cell lines. We showed that miRNA199a was significantly down regulated in HCC and non-HCC lines, while remaining high in primary hepatocytes, HepRG cells and Hepa1-6. The observation that murine Hepa1-6 cell line contains miRNA199a at levels comparable to hepatocytes allowed us to use it as a model for normal hepatocyte expression. These results were in line with other studies showing miRNA199a down regulation in HCC but expression at high levels in the cells of the liver including hepatocytes [17, 18], hepatic stellate cells[19] and liver sinusoid endothelial cells [20]. It has been reported as one of the key miRNAs dysregulated in HCC development and progression with a role as a diagnostic marker [21, 22] as well as a therapeutic target [23, 24]. miRNA199a has also been shown to be downregulated in a number of non-liver cancers such as breast cancer [25] renal cell cancer [26] osteosarcoma [27],thyroid cancer [28] and bladder cancer [29].

In line with previous studies performed with liver-specific miRNA122a [30, 31], transfection of reporters with three miRNA199a binding sites at the 3'-UTR resulted in a significant inhibition of the reporter expression in miRNA199a positive primary hepatocytes HepaRG, and Hepa1-6 cells while no significant effects were observed in miRNA199a negative HCC and non-HCC cell lines. No effect in expression was seen when a control miRNA binding site was incorporated at the 3'-UTR (supplementary Figure 1). Next, we generated plasmids harbouring cytosine deaminase (CD) and miRNA199a binding sites and observed significantly reduced cell death in miRNA199a positive cell Hepa1-6 after transfection and incubation with the prodrug 5-FC while in miRNA199a negative HCC cells, cell death

equivalent to CMV driven CD expression was observed. These results validated a post-transcriptionally targeted suicide gene therapeutic system for HCC. Given the complete homology between murine and human miRNA199a [32], we were able to model the effects of incorporating binding sites of miRNA199a at the 3'-UTR using Hepa1-6 which was useful especially for targeted GDEPT studies which is often problematic in primary hepatocytes due to culture conditions. We chose to use perfectly complementary binding sites as imperfectly complementary binding sites can cause saturation of endogenous miRNA even at low concentration of transcripts [15, 33], similarly, utilizing perfectly complementary binding induces target RNA degradation facilitating quick turnover [34, 35].

Next, we explored the possibility of constructing a targeted AAV vector harbouring our miRNA199a based post-transcriptionally regulated gene therapeutic system. Similar to reports by Peruta *et al.*, who utilized the liver specific nature of AAV8 to construct miRNA122a based post-transcriptionally liver detargeted gene delivery system, we observed that transduction of Hepa1-6 with scAAV8-GLuc-miR199a*3 leads to a significant reduction of reporter expression when compared to scAAV8-GLuc [36]. This observation was further corroborated by post-transcriptionally detargeted suicide gene therapy with scAAV8-CD-miR199a*3 in Hepa1-6. Given the ability of AAV8 to transduce liver and tissues of liver origin, our system could provide an option to target disseminated tumours.

In an interesting observation, we observed reduced levels of miRNA199a in 3D Hepa1-6 tumorspheres of enriched in stemness markers including CD44. This observation is in line with the reported tumor suppressive role of miRNA199a in HCC and the fact that miRNA199a directly regulates the expression of stemness marker CD44 in other cancers [37, 38]. While we have not directly shown the existence of cancer stem cells (CSC) in this study, others have shown that tumorspheres derived from HCC are enriched in stem like cells and exhibit high chemoresistance [39]. The potential utility of miRNA199 to target a sub-population

of CSCs is intriguing and could be explored in future studies. Although this particular study doesn't answer the question of how and what roles miRNA199a might play in HCC stem cell biology, the decreased levels of miRNA199a in Hepa1-6 tumorspheres allowed us to not only assess the effectiveness of miRNA199a targeting in a miRNA199a negative cell population but also showed its effectiveness in terms of increased expression and increased death when used in conjunction with GDEPT in a complex 3D tumor-like environment. Similar 3D tumoursphere culture systems are widely used to recapitulate some of the tumor heterogeneity seen *in vivo*, and to screen for novel drug candidates while reducing the need of animal models [40, 41] .

In conclusion, this proof-of-concept study establishes negative targeting based on post-transcriptional gene regulation by miRNA199a in the context of HCC gene therapy. This system was found to efficiently target HCC cells with downregulation of miRNA199a while at the same time detargeting miRNA199a positive HepaRG and Hepa1-6. Furthermore, AAV based delivery of this system was found to be feasible and effective. Finally, given that miRNA199a has been reported to be downregulated in multiple cancer types, this system could be exploited to detarget any cell type with high endogenous levels of miRNA199a.

Materials and Methods

Cell culture

Hepa1-6 cell line which expresses high levels of miRNA199a and HCC cell lines Hep3B, PLC/PRF/5, SKHep1, and SNU423 with miR199a downregulation were obtained from ATCC and maintained in DMEM media (Thermo Fisher Scientific, Scoresby, Australia) supplemented with 10% fetal bovine serum (FBS) (Gibco, Australia) and 1% penicillin/streptomycin (P/S) (Gibco, Australia). Australian Genome Research Facility (AGRF) cell line ID service was used to confirm the identity of the human cell lines. Breast

cancer cell lines T47D and MCF-7 were maintained in standard DMEM media. Melanoma cell lines 92.1, and Mel270 (gifted by Nicholas Hayward) and ovarian cancer lines SW626, CAOV3, TOV21G were grown in RPMI media (Thermo Fisher Scientific) supplemented with 10% FBS and 1% P/S. Prostate cancer cell lines LnCap and DU145 were maintained in standard DMEM media. All the other cell lines were maintained as per the ATCC recommendations. Cryopreserved primary human hepatocytes (HUM4150) and NoSpin HepaRG (NSHPRG) cells were obtained from Lonza, Australia and maintained as per the manufacturer's protocol.

Quantitative RT-PCR and quantification of miRNA levels

To quantify the endogenous expression levels of miRNA199a, total RNA was isolated with trizol and cDNA was synthesized with the MystiCq microRNA cDNA Synthesis Mix (Sigma Aldrich, St. Louis, MO, United States) as per the manufacturer's protocol. The synthesized cDNA was then used for quantitative real time PCR (qRT-PCR) with Bioline Lo-Rox Sybr (Bioline, Alexandria, Australia) in the ViiA7 RT-PCR machine (Thermo Fisher Scientific) at the following conditions: 95°C- 10mins followed by 40 cycles of 95°C- 5s, 60°C -10s and 70°C -10s. The MystiCq Universal PCR (MIRUP, Sigma) and 5'-CCCAGTGTTCAGACTACCTG- 3' primers were used to amplify miRNA199a and the amount of miR199a was calculated as number of copies per 1000 copies of RNU6 (MIRCP00001) control using the formula (2^(Ct control-Ct sample))*1000. A 100% homologous nature of murine and human miRNA199a allowed usage of the same primer for amplification. Similarly, markers for the level of stemness (CD44, CD133, and Oct4) were measured relative to GAPDH control (primers listed in supplementary table 1).

Construction of expression plasmids

The gene encoding GLuc with three miRNA199a-5p binding sites (GGGTCACAAGTCTGATGGACAAG*3) at the 3'-UTR was artificially synthesized (Thermo Fisher Scientific). The gene with and without miRNA binding sites was then cloned in the pscAAV-GFP (a gift from John T Gray, Addgene plasmid # 32396) using enzymes EcoRI, StuI and EcoRI, EcoRV to obtain CMV-GLuc and CMV-GLuc-miR199a*3 respectively. For the construction of cytosine deaminase (CD) expressing plasmids, the gene was artificially synthesized separately and cloned in the above-mentioned plasmids replacing GLuc to obtain CMV-CD and CMV-CD-miR199a*3. A control miRNA binding site GGGTCACAAGTCTGATGGACAAG *3 was also incorporated at the 3'-UTR of reporters (Supplementary figure 1). A representation of plasmid construction has been included in **Figure 5 (a)**.

Transfection and gaussia luciferase reporter assays

All transfection studies for investigating the reporter expression were performed with Lipofectamine 3000 (Thermo Fisher Scientific) in a 24 well plate as per the manufacturer's protocol. Briefly, 30,000 cells were seeded in a 24 well plate and transfection was performed with 500ng of plasmids. 72 hours post-transfection, the amount of GLuc secreted in the media was quantified with the Pierce gaussia luciferase glow assay kit (Thermo Fisher Scientific) as per the manufacturer's recommendations. The chemiluminescence measurement was done with the Infinite 200 Pro NanoQuant (Tecan Trading AG, Switzerland). In order to regulate the difference in transfection efficiencies across cell lines, chemiluminescence detected with CMV-GLuc-miR199a*3 was normalized with CMV-GLuc for individual cell type.

Inhibition and overexpression of miR199a

For knockdown experiments in Hepa1-6 cells, 5 pmol of miRNA199a inhibitor (4464084, Life Technologies, Mulgrave, Australia) was co-transfected using Lipofectamine 3000 with either CMV-GLuc or CMV-GLuc-199a*3 and either CMV-eGFP or CMV-eGFP-miR199a*3 in a 24 well plate as per the manufacturer's protocol. Similarly, for overexpression, miRNA199a mimic (4464066, Life Technologies) was co-transfected with either CMV-GLuc or CMV-GLuc-miR199a*3. 72 hours post transfection, percentage of GFP positive cells or secreted GLuc was quantified for each group and expressed as percentage of either CMV-eGFP or CMV-GLuc respectively.

Cell proliferation assay

Cell proliferation assay was performed with the CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI USA). Briefly, 10,000 cells were seeded in a 96 well plate and transfected with either CMV-CD or CMV-CD-199a*3. 24 hours post-transfection, fresh media containing 10µm 5-FC was added. After 48 hours, the cells were incubated with MTS reagent as per manufacturer's protocol and absorbance was measured at 540nm with the Infinite 200 Pro NanoQuant. Percentage proliferation was calculated for CMV-CD and CMV-CD-199a*3 for each cell line and percentage proliferation with CMV-CD-199a*3 was subtracted from that with CMV-CD to account for the difference in transfection efficiencies.

Cell death assay with Annexin V/PI

In order to quantify the amount of cell death after the expression of the suicide gene CD with or without miRNA199a binding site at the 3'-UTR, flow cytometry based on Annexin V/PI staining (Life Technologies) was performed as suggested by the supplier. Briefly, 30,000 cells were transfected with CMV-CD or CMV-CD-199a*3 in a 24-well plate. 48 hours post transfection, the media was replaced with fresh media containing 10 µM 5-FC. After 24

hours, the cells were washed, collected and stained with Annexin V/PI. Annexin V/PI positive cells were quantified using the FACS Canto II and data analysis was performed with FCS express 3 (BD Biosciences; North Ryde, Australia). The percentage of apoptotic cells after transfection with CMV-CD-199a*3 was normalized with CMV-CD for each cell type.

Adeno-associated virus production and transduction

AAV serotype 8 was produced using the triple transfection method using polyethylenimine (PEI). Briefly, HEK293 cells were seeded in 15 cm plates and transfected with pHelper (Agilent Technologies, Mulgrave, Australia), AAV8 capsid and either psc-CMV-GLuc, psc-CMV-GLuc-miR199*3, psc-CMV-CD, psc-CMV-CD-miR199*3 in a 2:1:1 ratio. After 48-72 hours, the cells were washed with PBS and subjected to 3 freeze-thaw cycles with ethanol/dry ice followed by incubation at 37°C to release the AAV. The crude lysate was then treated with benzonase (Sigma Aldrich), passed through the Amicon Ultrafilter 100 kDa (Sigma Aldrich) and buffer exchange was performed three times with PBS following which the end product was filtered through 0.22-micron filters. The number of vector genomes (vgs) was quantified with quantitative PCR (primers have been listed in **supplementary table 1**). All transduction experiments to study GLuc expression were performed in 96 well plates at an MOI of 100,000 vgs/cells and luminescence was measured as previously described. Similarly, suicide gene therapy was performed with an MTS assay after transduction of 10,000 cells with 100,000 vgs/cells of either pscAAV-CMV-CD or pscAAV-CMV-CD-miR199a*3 as previously described.

3D culture of Hepa1-6

3D tumorspheres of Hepa1-6 were maintained in stem cell conditioned, serum free NSA media containing DMEM/F12 (Thermo Fisher Scientific), 10ng/ml recombinant human basic fibroblast growth factor (rhFGF) (Lonza), 20ng/ml recombinant human epidermal growth

factor (rhEGF) (Lonza), bovine serum albumin (BSA) (Sigma Aldrich), 4µg/ml heparin sulfate (Sigma Aldrich), and 1% P/S (Thermo Fisher Scientific) as previously described [42]. Briefly, 30,000 cells were collected, washed thrice with PBS and seeded in ultra-low attachment plates (Corning, NY, USA). Images of tumorspheres were taken with a digital camera (Olympus DP21, Japan) connected to an inverted microscope (Olympus CKX41) with imaging software (CellSens, Olympus, Japan). Cells were either collected for RNA extraction (day 5), transfected at day 3 (with CMV-GLuc or CMV-GLuc-miR199a*3), or transduced at day 3 (with pscAAV8-GLuc) or pscAAV8-GLuc-miR199a*3).

Statistical analysis

All experiments were repeated at least thrice, and data represented as mean ± SD. To test whether there were significant differences in experimental results between groups, two tailed t-test was performed with Graph Pad Prism 7.0 (GraphPad Software, Inc.) (* <0.05, ** <0.01, ***<0.001).

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Author Contributions

B.D. and J.C.S. conceived and designed the experiments; B.D and CARS conducted the experiments and wrote the manuscript; J.C.S and C.J.L. edited the manuscript.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin* 2017.

2. Bupathi M, Kaseb A, Meric-Bernstam F, Naing A. Hepatocellular carcinoma: Where there is unmet need. *Mol Oncol* 2015.

3. Cidon EU. Systemic treatment of hepatocellular carcinoma: Past, present and future. *World J Hepatol* 2017; **9**(18)**:** 797-807.

4. Gao JJ, Shi ZY, Xia JF, Inagaki Y, Tang W. Sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma. *World J Gastroenterol* 2015; **21**(42)**:** 12059-70.

5. Wang YG, Huang PP, Zhang R, Ma BY, Zhou XM, Sun YF. Targeting adeno-associated virus and adenoviral gene therapy for hepatocellular carcinoma. *World J Gastroenterol* 2016; **22**(1)**:** 326-37.

6. Dhungel B, Jayachandran A, Layton CJ, Steel JC. Seek and destroy: targeted adeno-associated viruses for gene delivery to hepatocellular carcinoma. *Drug Deliv* 2017; **24**(1)**:** 289-299.

7. Liu Y, Siriwon N, Rohrs JA, Wang P. Generation of Targeted Adeno-Associated Virus (AAV) Vectors for Human Gene Therapy. *Curr Pharm Des* 2015; **21**(22)**:** 3248-56.

8. Chiba T, Iwama A, Yokosuka O. Cancer stem cells in hepatocellular carcinoma: Therapeutic implications based on stem cell biology. *Hepatol Res* 2016; **46**(1)**:** 50-7.

9. Foka P, Pourchet A, Hernandez-Alcoceba R, Doumba PP, Pissas G, Kouvatsis V *et al.* Novel tumour-specific promoters for transcriptional targeting of hepatocellular carcinoma by herpes simplex virus vectors. *J Gene Med* 2010; **12**(12)**:** 956-67.

10. Robson T, Hirst DG. Transcriptional Targeting in Cancer Gene Therapy. *J Biomed Biotechnol* 2003; **2003**(2)**:** 110-137.

11. Brown BD, Venneri MA, Zingale A, Sergi Sergi L, Naldini L. Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* 2006; **12**(5)**:** 585-91.

12. Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A *et al.* Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 2007; **25**(12)**:** 1457-67.

13. Xie J, Xie Q, Zhang H, Ameres SL, Hung JH, Su Q *et al.* MicroRNA-regulated, systemically delivered rAAV9: a step closer to CNS-restricted transgene expression. *Mol Ther* 2011; **19**(3)**:** 526-35.

14. Dhungel B, Ramlogan-Steel CA, Layton CJ, Steel JC. miRNA122a regulation of gene therapy vectors targeting hepatocellular cancer stem cells. *Oncotarget* 2018; **9**(34)**:** 23577-23588.

15. Dhungel B, Ramlogan-Steel CA, Steel JC. MicroRNA-Regulated Gene Delivery Systems for Research and Therapeutic Purposes. *Molecules* 2018; **23**(7).

16. Jayachandran A, Shrestha R, Dhungel B, Huang IT, Vasconcelos MYK, Morrison BJ *et al.* Murine hepatocellular carcinoma derived stem cells reveal epithelial-to-mesenchymal plasticity. *World J Stem Cells* 2017; **9**(9)**:** 159-168.

17. Li Y, Di C, Li W, Cai W, Tan X, Xu L *et al.* Oncomirs miRNA-221/222 and Tumor Suppressors miRNA-199a/195 Are Crucial miRNAs in Liver Cancer: A Systematic Analysis. *Dig Dis Sci* 2016; **61**(8)**:** 2315-27.

18. Jia XQ, Cheng HQ, Qian X, Bian CX, Shi ZM, Zhang JP *et al.* Lentivirus-mediated overexpression of microRNA-199a inhibits cell proliferation of human hepatocellular carcinoma. *Cell Biochem Biophys* 2012; **62**(1)**:** 237-44.

19. Murakami Y, Toyoda H, Tanaka M, Kuroda M, Harada Y, Matsuda F *et al.* The progression of liver fibrosis is related with overexpression of the miR-199 and 200 families. *PLoS One* 2011; **6**(1)**:** e16081.

20. Szabo G, Bala S. MicroRNAs in liver disease. *Nat Rev Gastroenterol Hepatol* 2013; **10**(9)**:** 542-52.

21. Ding Y, Yan JL, Fang AN, Zhou WF, Huang L. Circulating miRNAs as novel diagnostic biomarkers in hepatocellular carcinoma detection: a meta-analysis based on 24 articles. *Oncotarget* 2017; **8**(39)**:** 66402-66413.

22. El-Abd NE, Fawzy NA, El-Sheikh SM, Soliman ME. Circulating miRNA-122, miRNA-199a, and miRNA-16 as Biomarkers for Early Detection of Hepatocellular Carcinoma in Egyptian Patients with Chronic Hepatitis C Virus Infection. *Mol Diagn Ther* 2015; **19**(4)**:** 213-20.

23. Zhan Y, Zheng N, Teng F, Bao L, Liu F, Zhang M *et al.* MiR-199a/b-5p inhibits hepatocellular carcinoma progression by post-transcriptionally suppressing ROCK1. *Oncotarget* 2017; **8**(40)**:** 67169-67180.

24. Hou J, Lin L, Zhou W, Wang Z, Ding G, Dong Q *et al.* Identification of miRNomes in human liver and hepatocellular carcinoma reveals miR-199a/b-3p as therapeutic target for hepatocellular carcinoma. *Cancer Cell* 2011; **19**(2)**:** 232-43.

25. Shin VY, Siu JM, Cheuk I, Ng EK, Kwong A. Circulating cell-free miRNAs as biomarker for triple-negative breast cancer. *Br J Cancer* 2015; **112**(11)**:** 1751-9.

26. Tsukigi M, Bilim V, Yuuki K, Ugolkov A, Naito S, Nagaoka A *et al.* Re-expression of miR-199a suppresses renal cancer cell proliferation and survival by targeting GSK-3β. *Cancer Lett* 2012; **315**(2)**:** 189-97.

27. Duan Z, Choy E, Harmon D, Liu X, Susa M, Mankin H *et al.* MicroRNA-199a-3p is downregulated in human osteosarcoma and regulates cell proliferation and migration. *Mol Cancer Ther* 2011; **10**(8)**:** 1337-45.

28. Minna E, Romeo P, De Cecco L, Dugo M, Cassinelli G, Pilotti S *et al.* miR-199a-3p displays tumor suppressor functions in papillary thyroid carcinoma. *Oncotarget* 2014; **5**(9)**:** 2513-28.

29. Ichimi T, Enokida H, Okuno Y, Kunimoto R, Chiyomaru T, Kawamoto K *et al.* Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int J Cancer* 2009; **125**(2)**:** 345-52.

30. Qiao C, Yuan Z, Li J, He B, Zheng H, Mayer C *et al.* Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. *Gene Ther* 2011; **18**(4)**:** 403-10.

31. Wang G, Dong X, Tian W, Lu Y, Hu J, Liu Y *et al.* Evaluation of miR-122-regulated suicide gene therapy for hepatocellular carcinoma in an orthotopic mouse model. *Chin J Cancer Res* 2013; **25**(6)**:** 646-55.

32. Liu T, Chen Q, Huang Y, Huang Q, Jiang L, Guo L. Low microRNA-199a expression in human amniotic epithelial cell feeder layers maintains human-induced pluripotent stem cell pluripotency via increased leukemia inhibitory factor expression. *Acta Biochim Biophys Sin (Shanghai)* 2012; **44**(3)**:** 197-206.

33. Gentner B, Schira G, Giustacchini A, Amendola M, Brown BD, Ponzoni M *et al.* Stable knockdown of microRNA in vivo by lentiviral vectors. *Nat Methods* 2009; **6**(1)**:** 63-6.

34. Rüegger S, Großhans H. MicroRNA turnover: when, how, and why. *Trends Biochem Sci* 2012; **37**(10)**:** 436-46.

35. Sanei M, Chen X. Mechanisms of microRNA turnover. *Curr Opin Plant Biol* 2015; **27:** 199-206.

36. Della Peruta M, Badar A, Rosales C, Chokshi S, Kia A, Nathwani D *et al.* Preferential targeting of disseminated liver tumors using a recombinant adeno-associated viral vector. *Hum Gene Ther* 2015; **26**(2)**:** 94-103.

37. Cheng W, Liu T, Wan X, Gao Y, Wang H. MicroRNA-199a targets CD44 to suppress the tumorigenicity and multidrug resistance of ovarian cancer-initiating cells. *FEBS J* 2012; **279**(11)**:** 2047-59.

38. Liu R, Liu C, Zhang D, Liu B, Chen X, Rycaj K *et al.* miR-199a-3p targets stemness-related and mitogenic signaling pathways to suppress the expansion and tumorigenic capabilities of prostate cancer stem cells. *Oncotarget* 2016; **7**(35)**:** 56628-56642.

39. Zhang XL, Jia Q, Lv L, Deng T, Gao J. Tumorspheres Derived from HCC Cells are Enriched with Cancer Stem Cell-like Cells and Present High Chemoresistance Dependent on the Akt Pathway. *Anticancer Agents Med Chem* 2015; **15**(6)**:** 755-63.

40. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. *Nat Protoc* 2009; **4**(3)**:** 309-24.

41. Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA. Multicellular tumor spheroids: an underestimated tool is catching up again. *J Biotechnol* 2010; **148**(1)**:** 3-15.

42. Morrison BJ, Steel JC, Morris JC. Sphere culture of murine lung cancer cell lines are enriched with cancer initiating cells. *PLoS One* 2012; **7**(11)**:** e49752.

Figure Legends

Figure 1: <u>Expression pattern of miRNA199a:</u> The expression levels of miRNA199a was measured by quantitative real time PCR and was performed in primary hepatocytes, HepaRG and a panel of HCC **(a)** and non-HCC cell lines **(b).** The copies of miRNA199a was normalized against that of the RNU6 control and represented as the number of copies per 1000 control. (n>3 for all cell lines except HUM4150)

Figure 2: <u>Reporter gene expression under the control of miRNA199a binding sites:</u> (a) Principle of miRNA mediated detargeting: Inclusion of binding sites of miRNA expressed at high levels in a cell can limit the expression of transgene in that particular cell while minimal effects is expected in cells that don't express the miRNA. **(b)** To study the effect of the presence of miRNA199a binding sites at the 3'-UTR of a transgene, miRNA199a positive cells HepaRG, and Hepa1-6 were transfected with CMV-GLuc and CMV-GLuc-miR199a*3; GLuc expression with CMV-GLuc-miR199a*3 was reported as a percentage of CMV-GLuc. Similarly, miRNA199a negative HCC cell lines **(c)** and non-HCC cell lines **(d)** were transfected with CMV-GLuc and CMV-GLuc-miR199a*3 and the amount of secreted GLuc was quantified. **(e)** Further confirmation of miRNA199a mediated gene regulation was performed by transfection of Hepa1-6 cells with CMV-eGFP and CMV-eGFP-miR199a*3;

572 72 hours post transfection, the percentage of GFP positive cells was quantified with flow
573 cytometry and the percentage of GFP positive cells after transfection with CMV-eGFP-
574 miR199a*3 was normalized with CMV-eGFP. Two tailed t-test was used for statistical
575 analysis to compare percentage expression of CMV-GLuc-miR199a*3 or CMV-eGFP-
576 miR199a*3 with CMV-GLuc or CMV-eGFP respectively for both HepaRG and Hepa1-6
577 using Graph Pad Prism 7.0. (n>3, ** p<0.005, *** p<0.001)

578 **Figure 3: <u>Overexpression and inhibition of miRNA199a and its effect on the expression</u>**
579 **<u>of reporters containing miRNA199a binding sites at the 3'-UTR:</u>** To investigate the
580 effects of inhibiting miRNA199a on the expression of reporter genes with binding site at the
581 3'-UTR, Hepa1-6 cells were co-transfected with either CMV-eGFP or CMV-eGFP-
582 miR199a*3 and miRNA199a inhibitor (**a**), and either CMV-GLuc or CMV-GLuc-
583 miR199a*3 and miRNA199a inhibitor (**c**). The percentage of GFP positive cells for each
584 group was quantified with flow cytometry and expressed as percentage of GFP positive cells
585 after transfection with CMV-eGFP. Similarly, the amount secreted luciferase was quantified
586 and expressed as a percentage of quantity of secreted luciferase after transfection with CMV-
587 GLuc for each treatment group. (**b**) Representative fluorescent microscope and flow
588 cytometry images for each treatment groups. (**d**) The effects of overexpressing miRNA199a
589 on reporter expression in HCC cell lines by co-transfecting either CMV-GLuc or CMV-
590 GLuc-miR199a*3 and miRNA199a mimic was studied. The secreted luciferase was
591 quantified and represented as percentage of secreted luciferase after transfection with CMV-
592 GLuc for each treatment group. Two tailed t-test was performed to compare the difference
593 between indicated groups using Graph Pad 7.0 (n>3, * p<0.05)

594 **Figure 4: <u>Targeted gene directed enzyme prodrug therapy (GDEPT) utilizing</u>**
595 **<u>miRNA199a binding site:</u> (a)** Cells were seeded in a 96-well plate and transfected with

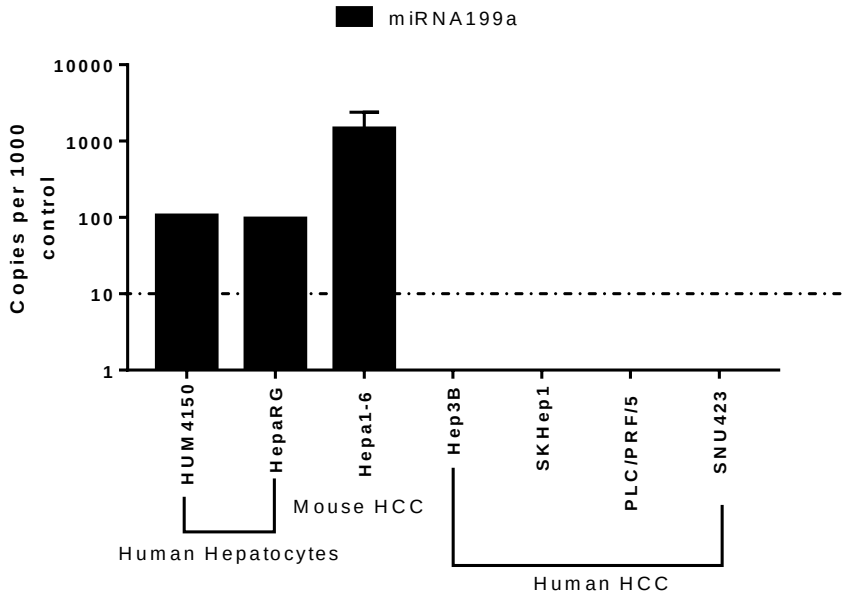
CMV-CD and CMV-CD-miR199a*3; after incubation with 5-FC, MTS assay was performed to quantify cell proliferation. Percentage proliferation with CMV-CD-199a*3 was normalized with CMV-CD for each cell line. **(b)** In order to quantify total cell death after GDEPT, cells were transfected in 24 well plates with CMV-CD and CMV-CD-miR199a*3 and incubated with media containing 5-FC. Percentage of apoptotic cells were then calculated by annexin/propidium iodide (PI) staining. Percentage death after GDEPT with CMV-CD-199a*3 was normalized against that with CMV-CD. **(c)** Representative flow cytometric analysis of Hepa1-6 showing the difference of apoptotic cells after GDEPT with CMV-CD, and CMV-CD-miR199a*3. **(d)** Representative flow cytometric images of miRNA199a positive Hepa1-6 and miRNA199a negative Hep3B, PLC/PRF/5, and SKHep1 showing percentage of annexin and PI positive cells after GDEPT with CMV-CD and CMV-CD-miR199a*3. Experiments were repeated thrice in triplicates and the difference between CMV-CD and CMV-CD-miR199a groups was checked for statistical significance using the two tailed t-test in Graph Pad Prism 7.0 (*$p < 0.05$ ***$p<0.005$)

Figure 5: <u>Adeno associated virus (AAV) vector system mediated targeted gene therapy based on post-transcriptional action of miRNA199a:</u> To investigate the possibility of vector mediated delivery of transgene harboring miR199a binding site, AAV8 harboring GLuc as reporter and cytosine deaminase (CD) as therapeutic gene was constructed with or without miR199a binding sites at the 3'-UTR of the transgenes and cells were transduced at an MOI of 100,000 vgs/cell. **(a)** Construction of plasmid harboring GLuc, and CD flanked by self-complementary inverted terminal repeats of the Adeno associated virus. **(b)** *Reporter expression after delivery with AAV*: Both miRNA199a positive and negative cells were transduced with scAAV8-GLuc and scAAV8-GLuc-miR199a*3 and the amount of secreted GLuc was quantified. Relative expression of GLuc after transduction with scAAV8-GLuc-miR199a*3 was reported as a percentage of that after transduction with scAAV8-GLuc. **(b)**

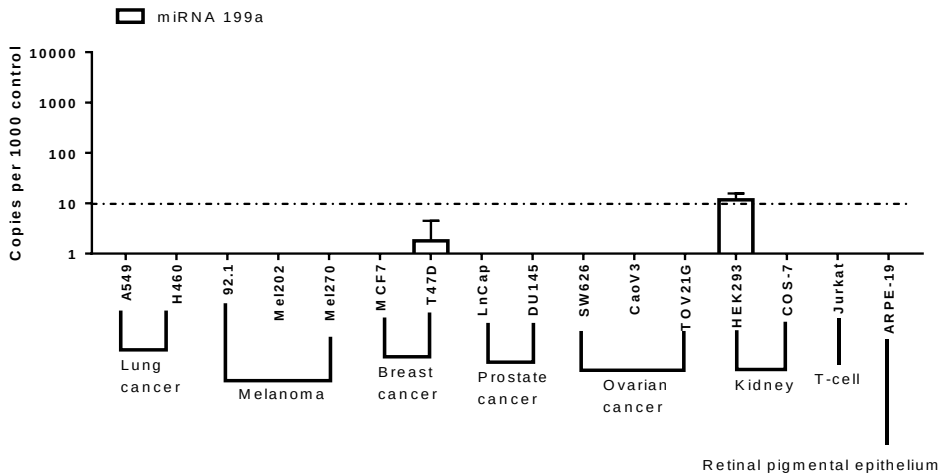
Targeted GDEPT after AAV mediated suicide gene therapy: Cells were transduced with scAAV8-CD, and scAAV8-CD-miR199a*3 and incubated with the prodrug 5-FC. The percentage proliferation was then calculated for scAAV8-CD-miR199a*3 and represented as a percentage of that for scAAV8-CD for each cell type. The significant difference between groups was tested by two-tailed t-test using Graph Pad prism 7.0. (n>3, *<0.05).

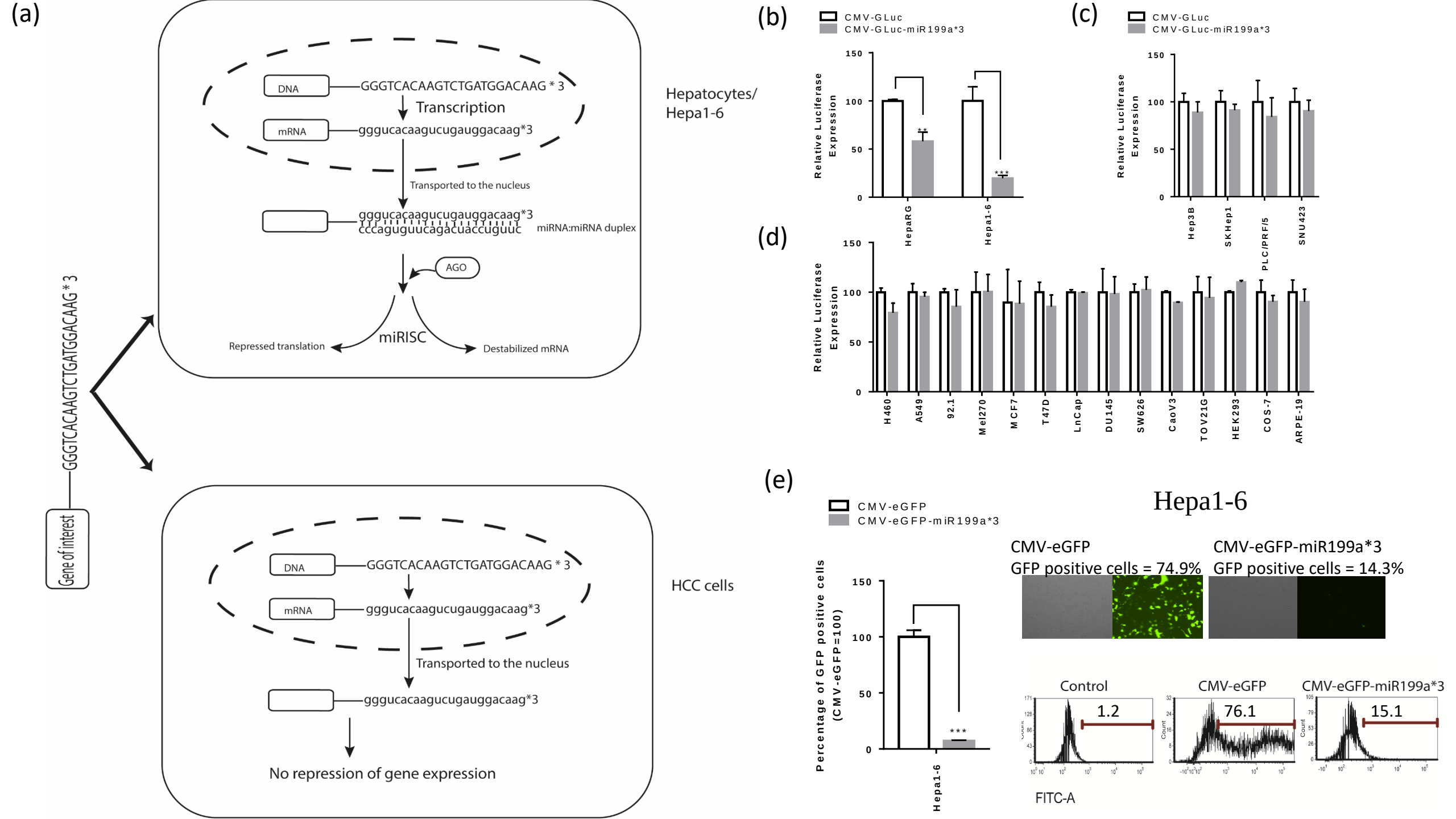
Figure 6: Expression of miRNA199a in stem cell enriched tumorspheres of Hepa1-6 and its implication for targeted gene delivery: (a) Photomicrographs of tumorspheres of Hepa1-6 after 5 days in culture in serum free, stem cell enriching media (scale bar = 50µM). Quantitative real time PCR was performed on cDNA of the Hepa1-6 and Hepa1-6 tumorspheres to compare the markers of stemness, CD44 **(b)**, CD133 **(c)**, and Oct4 **(d)** as well as miRNA199a **(e)** Comparison of miRNA199a levels in Hepa1-6 and Hepa1-6 tumorspheres. **(f)** Hepa1-6 and Hepa1-6 tumorspheres were transfected with CMV-GLuc-miR199a*3 and secreted GLuc was reported as percentage of CMV-GLuc **(g)** Hepa1-6 and Hepa1-6 tumorspheres were transduced with pscAAV8-GLuc and pscAAV8-GLuc-miR199a*3 and relative GLuc expression was reported. (**h**) Similarly, Hepa1-6 and Hepa1-6 tumorspheres were transfected with CMV-CD and CMV-CD-miR199a*3 and percentage proliferation was calculated following incubation with 5-FC. Percentage proliferation for CMV-CDmiR199a*3 was normalized with CMV-CD. Two tailed t-test was performed to analyze the difference between groups. (n>3, *<0.05, **<0.01).

(a)

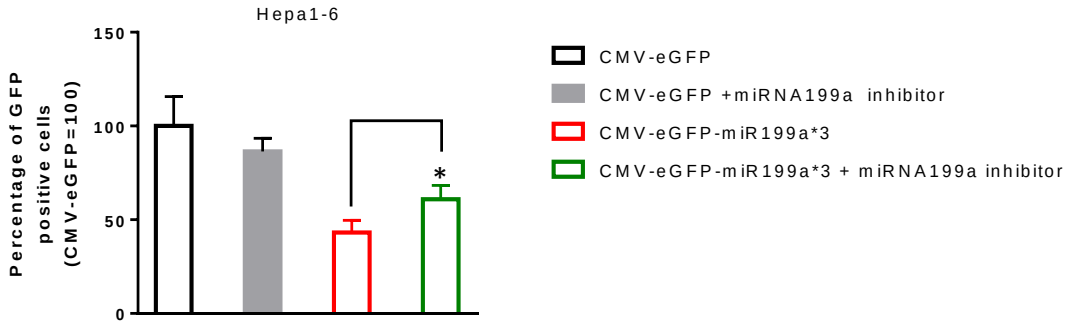

miRNA199a
Copies per 1000 control
10000
1000
100
10
1
HUM4150
HepaRG
Hepa1-6
Hep3B
SKHep1
PLC/PRF/5
SNU423
Mouse HCC
Human Hepatocytes
Human HCC

(b)

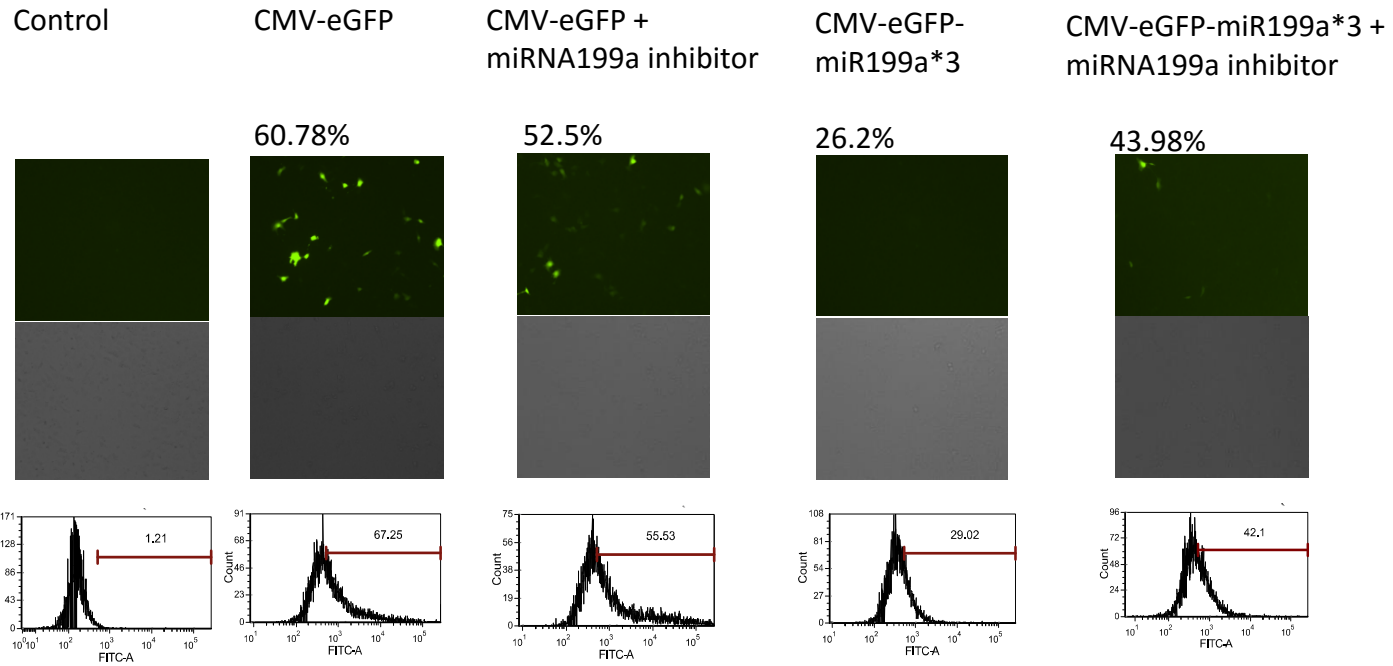

miRNA 199a
Copies per 1000 control
10000
1000
100
10
1
A549
H460
92.1
Mel202
Mel270
MCF7
T47D
LnCap
DU145
SW626
CaoV3
TOV21G
HEK293
COS-7
Jurkat
ARPE-19
Lung cancer
Melanoma
Breast cancer
Prostate cancer
Ovarian cancer
Kidney
T-cell
Retinal pigmental epithelium


(a)
GGGTCACAAGTCTGATGGACAAG * 3
Gene of interest
DNA
GGGTCACAAGTCTGATGGACAAG * 3
Transcription
mRNA
gggucacaagucugauggacaag*3
Transported to the nucleus
gggucacaagucugauggacaag*3
cccaguguucagacuaccuguuc
miRNA:miRNA duplex
AGO
miRISC
Repressed translation
Destabilized mRNA
Hepatocytes/
Hepa1-6
DNA
GGGTCACAAGTCTGATGGACAAG * 3
mRNA
gggucacaagucugauggacaag*3
Transported to the nucleus
gggucacaagucugauggacaag*3
No repression of gene expression
HCC cells
(b)
CMV-GLuc
CMV-GLuc-miR199a*3
Relative Luciferase Expression
150
100
50
0
**
***
HepaRG
Hepa1-6
(c)
CMV-GLuc
CMV-GLuc-miR199a*3
Relative Luciferase Expression
150
100
50
0
Hep3B
SKHep1
PLC/PRF/5
SNU423
(d)
Relative Luciferase Expression
150
100
50
0
H460
A549
92.1
Mel270
MCF7
T47D
LnCap
DU145
SW626
CaoV3
TOV21G
HEK293
COS-7
ARPE-19
(e)
CMV-eGFP
CMV-eGFP-miR199a*3
Percentage of GFP positive cells (CMV-eGFP=100)
150
100
50
0
***
Hepa1-6
Hepa1-6
CMV-eGFP
GFP positive cells = 74.9%
CMV-eGFP-miR199a*3
GFP positive cells = 14.3%
Control
CMV-eGFP
CMV-eGFP-miR199a*3
1.2
76.1
15.1
Count
FITC-A

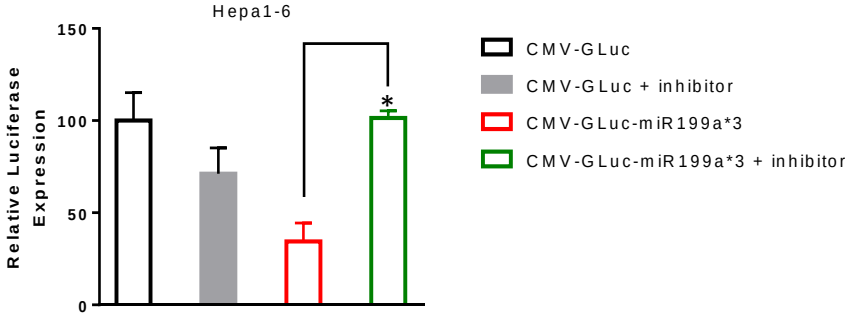
(a)


Hepa1-6
Percentage of GFP positive cells (CMV-eGFP=100)
150
100
50
0
*
CMV-eGFP
CMV-eGFP +miRNA199a inhibitor
CMV-eGFP-miR199a*3
CMV-eGFP-miR199a*3 + miRNA199a inhibitor

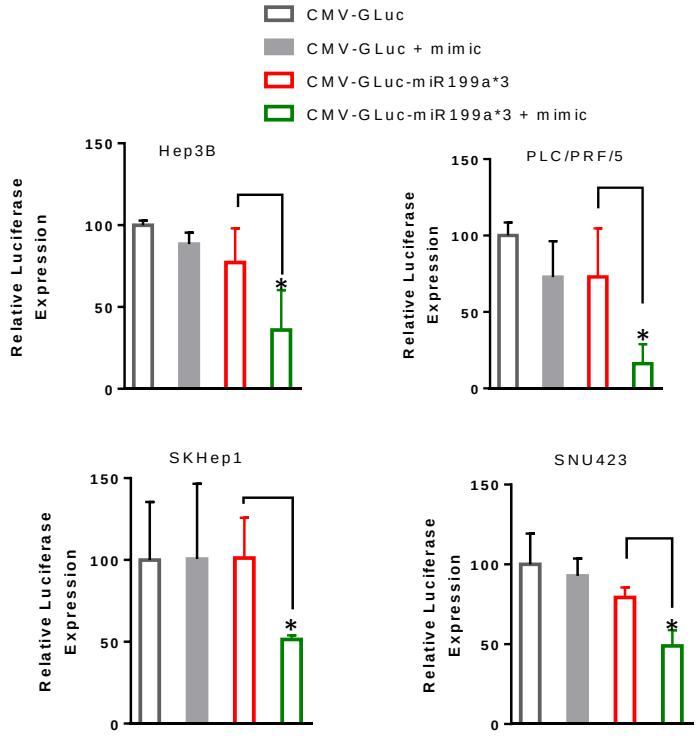
(b)


Control
CMV-eGFP
CMV-eGFP + miRNA199a inhibitor
CMV-eGFP-miR199a*3
CMV-eGFP-miR199a*3 + miRNA199a inhibitor
60.78%
52.5%
26.2%
43.98%
1.21
67.25
55.53
29.02
42.1
Count
FITC-A

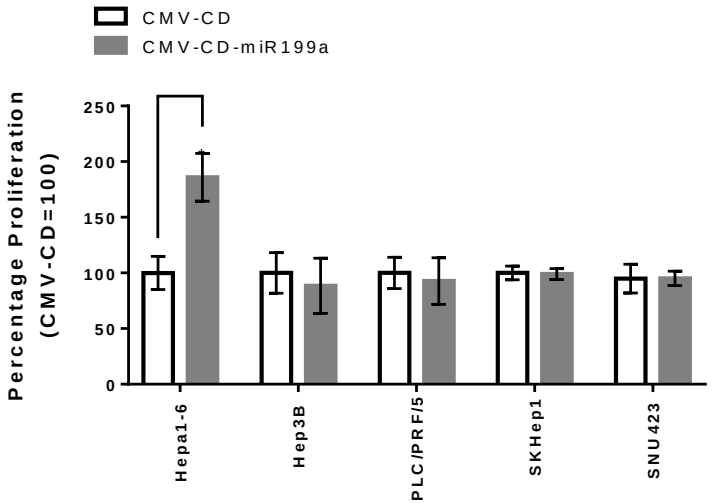
(c)


Hepa1-6
Relative Luciferase Expression
150
100
50
0
*
CMV-GLuc
CMV-GLuc + inhibitor
CMV-GLuc-miR199a*3
CMV-GLuc-miR199a*3 + inhibitor

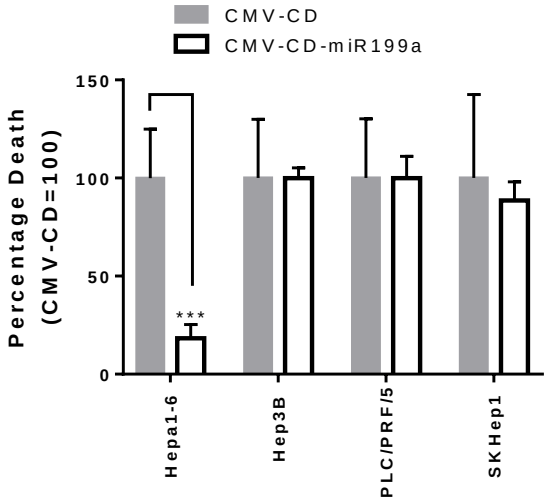
(d)


CMV-GLuc
CMV-GLuc + mimic
CMV-GLuc-miR199a*3
CMV-GLuc-miR199a*3 + mimic
Hep3B
PLC/PRF/5
SKHep1
SNU423
Relative Luciferase Expression
150
100
50
0
*

(a)

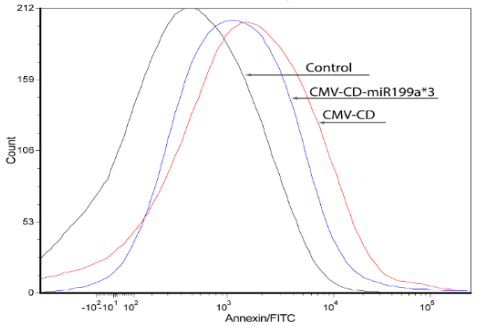

CMV-CD
CMV-CD-miR199a
Percentage Proliferation (CMV-CD=100)
250
200
150
100
50
0
Hepa1-6
Hep3B
PLC/PRF/5
SKHep1
SNU423

(b)

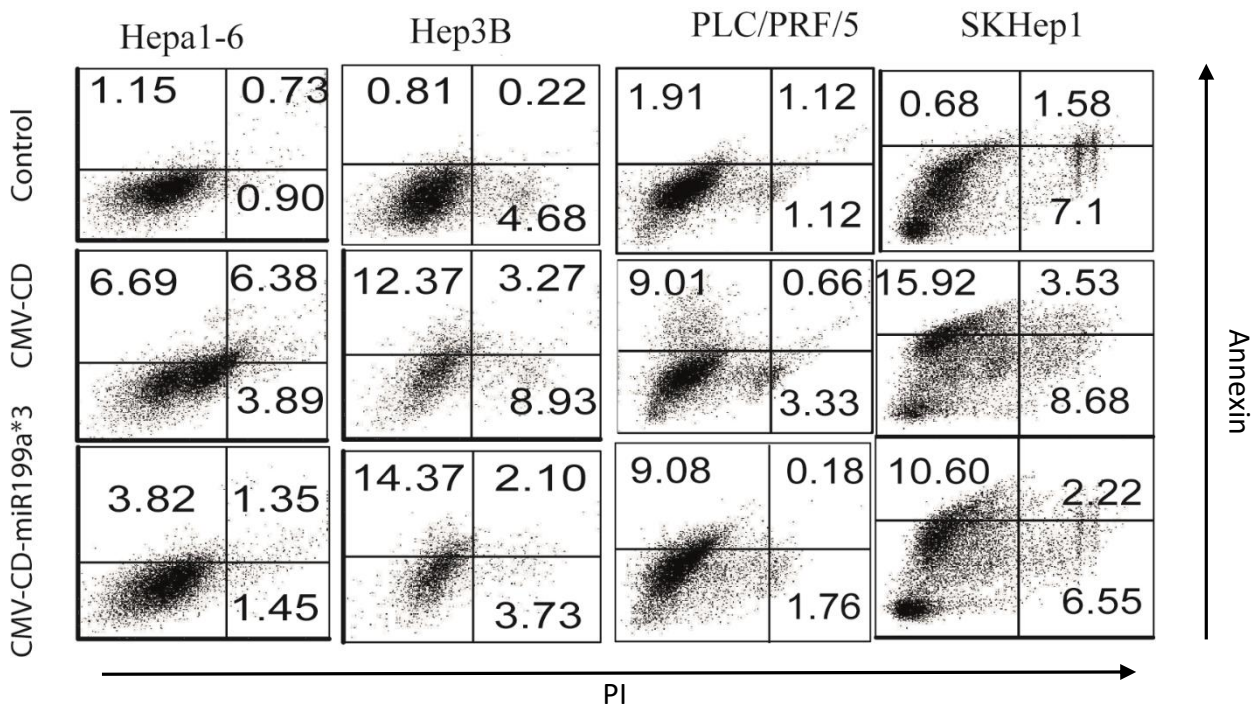

CMV-CD
CMV-CD-miR199a
Percentage Death (CMV-CD=100)
150
100
50
0
***
Hepa1-6
Hep3B
PLC/PRF/5
SKHep1

(c)

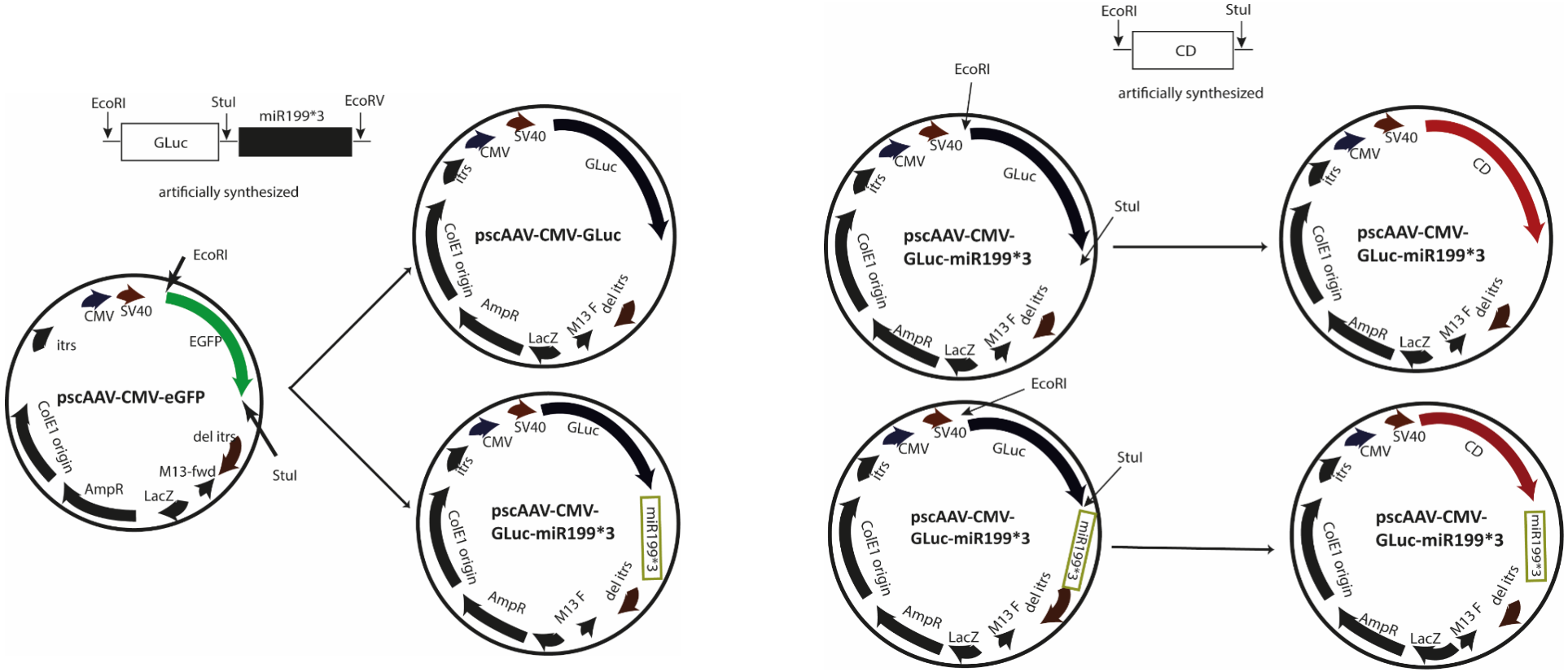
Hepa1-6


212
159
106
53
0
Count
Control
CMV-CD-miR199a*3
CMV-CD
Annexin/FITC

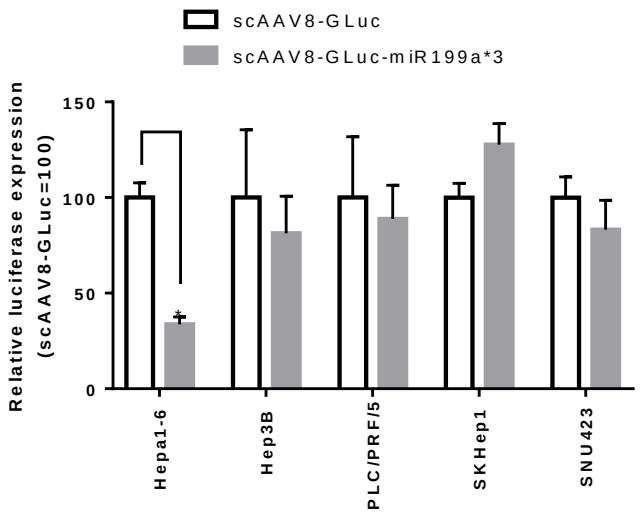
(d)


Hepa1-6
Hep3B
PLC/PRF/5
SKHep1
Control
CMV-CD
CMV-CD-miR199a*3
1.15
0.73
0.90
0.81
0.22
4.68
1.91
1.12
1.12
0.68
1.58
7.1
6.69
6.38
3.89
12.37
3.27
8.93
9.01
0.66
3.33
15.92
3.53
8.68
3.82
1.35
1.45
14.37
2.10
3.73
9.08
0.18
1.76
10.60
2.22
6.55
Annexin
PI

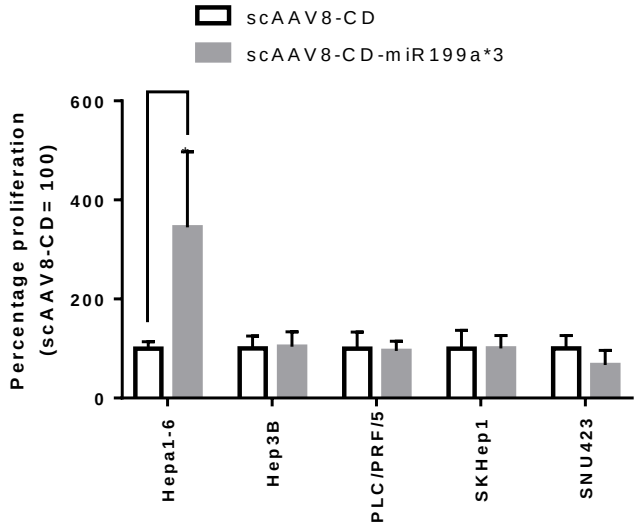
(a)


EcoRI
StuI
miR199*3
EcoRV
GLuc
artificially synthesized
pscAAV-CMV-eGFP
CMV
SV40
itrs
EGFP
ColE1 origin
del itrs
M13-fwd
AmpR
LacZ
pscAAV-CMV-GLuc
GLuc
M13 F
pscAAV-CMV-GLuc-miR199*3
CD
artificially synthesized

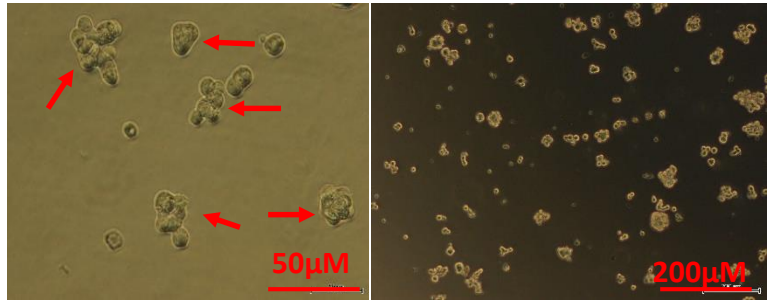
(b)


scAAV8-GLuc
scAAV8-GLuc-miR199a*3
Relative luciferase expression
(scAAV8-GLuc=100)
150
100
50
0
Hepa1-6
Hep3B
PLC/PRF/5
SKHep1
SNU423

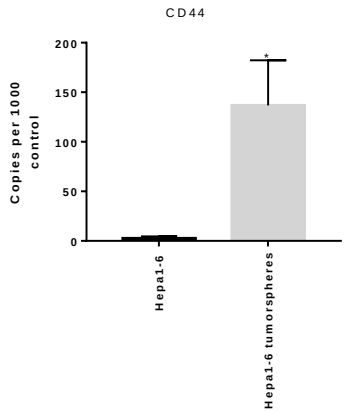
(c)


scAAV8-CD
scAAV8-CD-miR199a*3
Percentage proliferation
(scAAV8-CD= 100)
600
400
200
0
Hepa1-6
Hep3B
PLC/PRF/5
SKHep1
SNU423

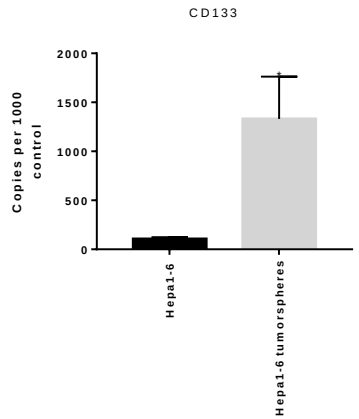
(a)


50μM
200μM

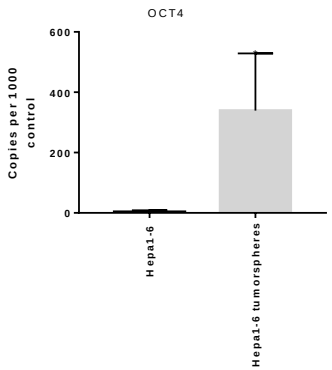
(b)


CD44
Copies per 1000 control
200
150
100
50
0
*
Hepa1-6
Hepa1-6 tumorspheres

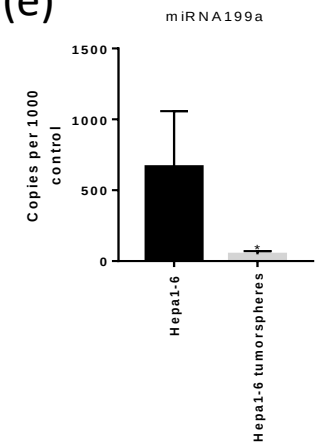
(c)


CD133
Copies per 1000 control
2000
1500
1000
500
0
*
Hepa1-6
Hepa1-6 tumorspheres

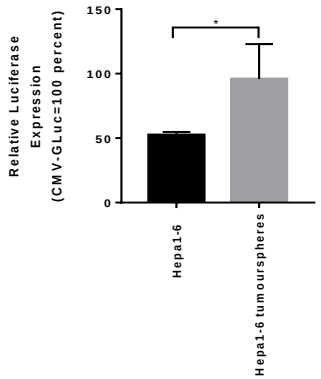
(d)


OCT4
Copies per 1000 control
600
400
200
0
Hepa1-6
Hepa1-6 tumorspheres

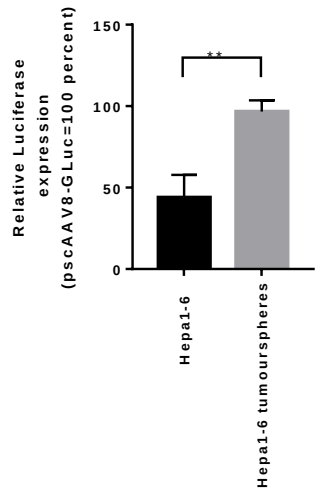
(e)


miRNA199a
Copies per 1000 control
1500
1000
500
0
*
Hepa1-6
Hepa1-6 tumorspheres

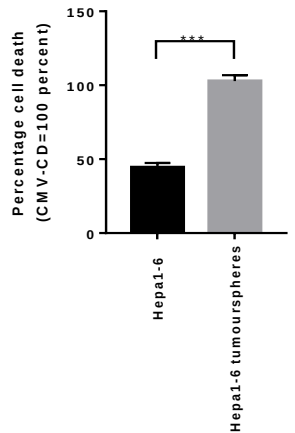
(f)


Relative Luciferase Expression (CMV-GLuc=100 percent)
150
100
50
0
*
Hepa1-6
Hepa1-6 tumourspheres

(g)


Relative Luciferase expression (pscAAV8-GLuc=100 percent)
150
100
50
0
**
Hepa1-6
Hepa1-6 tumourspheres

(h)


Percentage cell death (CMV-CD=100 percent)
150
100
50
0
***
Hepa1-6
Hepa1-6 tumourspheres