TITLE PAGE**MicroRNA-342-3p is a regulator of hepatocellular carcinoma regression.**

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Abstract

Background & Aims: Hepatocellular carcinoma (HCC) is a cancer with multiple etiologies, and widespread prevalence. Largely refractory to current treatments, HCC is the fourth leading cause of cancer related deaths, world-wide. MicroRNAs (miRNAs) are important regulators in HCCs. We aimed to identify tumor suppressor miRNAs during tumor regression in a conditional c-MYC driven mouse model (LT2/MYC) of HCC, and to evaluate their therapeutic potential for HCC treatment. Methods: We performed miRNA expression profiling of developed and regressing LT2/MYC tumors, and in-depth *in vitro* gain- and loss-of-function analyses. Effect of adeno-associated virus vector (AAV)-mediated miR-342-3p treatment was evaluated in three HCC mouse models. Results: We identified miR-342-3p as a tumor suppressor miRNA in HCC, with increased expression in regressing tumors. Forced miR-342-3p expression in hepatoma cells showed significantly decreased cell proliferation, migration and colony formation. *In vivo* administration of AAV-miR-342-3p led to significant attenuation of tumor development and increased overall survival. We identified *Mct1* as a *bona fide* target of miR-342-3p in HCC. We show the tumor suppressor role of miR-342-3p is executed partly by modulating the lactate transport function of MCT1. Importantly, we find miR-342-3p downregulated in tumors from HCC patients compared to matched non-tumor tissues, inversely correlating with MCT1 expression. We observed similar findings in TCGA-LIHC data. Conclusion: In our study, we identified and validated miR-342-3p as a tumor suppressor miRNA, in HCC. We demonstrated its therapeutic efficacy in significantly attenuating tumor development, and prolonging survival, in different HCC mouse models. Identification of miR-342-3p as an effective tumor suppressor opens a therapeutic avenue for miRNA-mediated attenuation of HCC development.

Lay Summary

Hepatocellular carcinoma (HCC), the most common type of liver cancer, affects diverse populations and has global impact, being the fourth leading cause of cancer deaths, worldwide. HCC, which cannot be surgically removed, has no effective therapies for long-term survival. Thus, novel effective treatment options are urgently required. To understand the molecular basis of how tumors regress, we compared tumors and regressing liver tumors in mice. We show that small non-coding microRNA, miR-342-3p is a tumor suppressor in HCC. Expression of miR-342-3p is low in tumors and high in regressing tumors. When miR-342-3p is delivered to mouse livers with HCC, it can significantly slow down liver tumor development and improve survival. Our study highlights the promising therapeutic potential of miR-342-3p intervention in HCC.

INTRODUCTION

HCC is currently, a largely intractable disease. Apart from early surgical resection and liver transplantation, no effective cures currently exist. Patients with advanced unresectable HCC may receive tyrosine kinase inhibitors, Sorafenib, Lenvatinib, (1, 2), PD-L1 inhibitor Atezolizumab, or monoclonal antibody Bevacizumab, for first-line therapy, and Regorafenib for second-line treatment(3). Despite these advances, existing therapeutics are limited in their activity against HCC, and novel treatments need to be developed.

The property of hepatocytes to readily take up small nucleic acids opens novel therapeutic avenues against HCC. For example, small non-coding microRNAs (miRNAs), which play important regulatory roles in cancer development, show promising potential for miRNA-based therapies in HCC (4, 5).

Since *c-MYC* is a central gene in HCC, we have previously (4, 6) used conditional doxycycline-regulated LT2/MYC mice, to study HCC. Hepatocyte specific activation of *c-MYC* by removal of doxycycline-containing diet, gives rise to liver tumors (4), which readily regress when mice are re-fed a doxycycline-containing diet to represses MYC expression (6),(7). The LT2/MYC model thus permits us to study molecular events during tumor formation and the earliest stages of tumor regression.

To gain further insight into miRNA-based regulatory mechanisms in HCC regression, and explore new opportunities for therapeutic interventions, we performed miRNA expression profiling of tumors and regressing liver tumors from LT2/MYC mice. Following in-depth *in silico*, *in vitro* and xenograft analyses, we confirmed miR-342-3p as a *bona fide* tumor suppressor in HCC. AAV-mediated *in vivo* delivery of miR-342-3p, in three independent mouse models of HCC, led to significant attenuation of tumor progression and prolonged survival. We identified and validated Solute Carrier Family 16, Member 1 / Monocarboxylic Acid Transporter 1 (SLC16A1 / MCT1), as a direct target of miR-342-3p and showed that miR-

342-3p-mediated MCT1 knockdown affects its lactate transport function, in HCC. Our study highlights the therapeutic potential of miR-342-3p as a tumor suppressor miRNA in the treatment of HCC.

MATERIALS AND METHODS

Mouse models

All animal experiments were performed in accordance with ethical guidelines on animal care, directed by the Hannover medical school (MHH) and the approved animal protocol. Animals were housed in the MHH-Twincore animal facility and maintained with access to water and standard or doxycycline-containing food, *ad libitum*. We used adult LT2/MYC, LT2/RAS, and 8-12 weeks old BALB/cAnNRj-Foxn1 nu/nu mice. All experimental animals were closely monitored for their general health status.

MicroRNA expression profiling

We performed global microRNA expression profiling on liver tumors and regressing tumors from LT2/MYC mice. Total RNA was extracted from tissues (mirVana™ miRNA Isolation Kit, ThermoFisher Scientific). RNA was DNase-treated (Ambion DNase I (RNase-free), ThermoFisher Scientific) and sent to the MicroRNA Array Services, Exiqon for quality control (Bioanalyser 2100) followed by miRCURY™ LNA Array microRNA profiling.

Gene expression profiling

DNase-treated total RNA from Huh7 cells treated with miR-342 mimic or Scramble control was sent to the Genome Analytics core facility at the Helmholtz Center for Infection Research, Braunschweig, Germany, for global mRNA microarray profiling with the Agilent Whole Human Genome Oligo Microarrays (G4112A) platform.

In silico analyses

Cluster and TreeView were used to perform hierarchical clustering and generate heat maps.

Gene set enrichment analyses (GSEA) on subsets of energy metabolism genes was performed using mRNA array data from miR-342-3p mimic and scramble treated Huh7 cells.

Survival probability from the TCGA-LIHC dataset was analyzed by Xena Functional Genomics Explorer resource (BioRxiv doi: <https://doi.org/10.1101/326470>). PCA plot showing separate clustering of tumor (T) and early regressing tumor (RT) global miRNA expression profiles was generated via Omicsoft, Oncoland array studio (trial version, Qiagen). Graphpad, Prism was used to generate most of the graphs and the Kaplan-Meier analyses. Microsoft Excel or Graphpad, Prism were used to calculate significance.

Adeno-associated virus serotype 8 (AAV8) production

293T cells were transfected with pDP8.ape (Plasmid Factory, Bielefeld, Germany) and pAAV-Ttr-Control or pAAV-Ttr-mir-342 plasmids using the calcium phosphate AAV8 production method established in our laboratory. Viral titers were determined by quantitative polymerase chain reaction (qPCR) using primers targeting the Ttr promoter.

Statistical analyses

Significance was determined with two-tailed Student's t test for comparison of 2 groups. All p <0.05 values were considered significant. The Kaplan-Meier analysis was used for survival studies. Log-rank (Mantel-Cox) Test was used to compare survival curves between the treatment groups. Error bars represent $\pm$ standard error of the mean (SEM).

Additional detailed methods are provided in Supplementary data and CTAT table.

RESULTS

MiRNA profiling reveals distinctly deregulated miRNAs during liver tumor regression and identifies miR-342-3p as a tumor suppressor

The LT2/MYC mouse model enables controlled liver tumor development and regression by liver specific activation or repression of *c-MYC* via doxycycline (**Supplementary Fig. S1A**). Significantly reduced tumor burden was seen within three days after *c-MYC* inactivation (6) and histological differences were observed between tumors and early regressing tumors (**Fig. 1 A**). To identify miRNAs, which regulate tumor regression, we performed miRNA expression profiling and found significant differential regulation of global miRNA expression profiles from established tumors (T) and regressing tumors (RT), 72h after suppression of *c-MYC*. (**Fig. 1 B and Supplementary Fig. S1B**). Based on a >2-fold differential regulation and a $p < 0.05$, we identified (**Fig. 1 C**) and validated by quantitative PCR (qPCR), four miRNAs whose expression profiles indicated a potential function in tumor regression, with increased expression in the RT group - miR-342-3p, miR-4651, miR-136-5p, and miR-22-3p (**Fig. 1 D**). We performed gain-of-function studies to analyze their effects on characteristic hallmarks of cancer. Basal endogenous levels of these miRNAs in human hepatoma cell lines Huh7, HepG2, HepaRG and SNU182 compared to normal human hepatocytes, was first determined. Huh7 showed the lowest endogenous expression of all miRNA candidates (**Supplementary Fig. S1 C**) and was thus used to establish stable expression of individual miRNAs with respective lentiviral vectors (LV-miR) (representative example in **Supplementary Fig. S2 A**). A corresponding control Huh7 cell line (LV_Control), expressed dTomato, but no miRNA. We found only Huh7 LV_342 cells, stably expressing miR-342-3p and a dTomato reporter, showed a consistent decrease in proliferation, 48h post cell seeding, with the WST-1 cell proliferation assay (**Fig. 1 E**), and, most robustly and reproducibly suppressed both colony

forming ability in soft agar assays (**Fig. 1 F**), and migration of cells in wound healing assays (**Fig. 1 G**), compared to LV_Control cells. Our miRNA expression profiling, validation in mouse liver tumors, and *in vitro* functional analyses, strongly support a tumor suppressor role for miR-342-3p, which was thus selected for further study.

MiR-342-3p suppresses growth of HCC cells, *in vitro* and in a xenograft tumor model

To determine whether growth inhibitory effects in LV_342 cells indeed resulted from specific gain of miR-342-3p, we inhibited miR-342-3p by transfecting LV_342 cells with a miR-342-3p inhibitor. MiR-342-3p inhibition was confirmed by qPCR (**Supplementary Fig. S2 B**). We found that miR-342-3p inhibitor treatment rescued inhibitory effects of miR-342-3p seen in comparison to LV_Control scramble-treated cells (**Fig. 2 A-C**). MiR-342-3p inhibition led to significant increase in cell proliferation with the WST-1 assay (**Fig. 2 A**), a higher number of proliferation marker, Ki67-positive cells by immunofluorescence staining (**Fig. 2 B**), increased number of colonies by soft agar colony forming assay (**Fig. 2 C**), and increased cell migration by wound healing assay (**Fig. 2 D, Supplementary Fig. S2 C**). Annexin V staining followed by FACS, showed significant increase in apoptosis in LV_342 Scr cells compared to LV_Control Scr cells, which significantly decreased following miR-342-3p inhibition (**Fig. 2 E**), indicating rescue of the pro-apoptotic effect of miR-342-3p. Hence, our *in vitro* gain- and loss-of-function experiments clearly indicate that miR-342-3p is a *bona fide* tumor suppressor miRNA.

To investigate whether miR-342-3p regulates tumorigenicity in a xenograft tumor model, we injected 1×10^6 LV_342 or LV_Control cells subcutaneously into flanks of female BALB/c Nu/Nu nude mice. Significant attenuation of tumor development and decreased tumor volumes and weights, were seen in LV_342 tumors (**Fig. 3 A-B**), which still showed high miR-342-3p expression (**Fig. 3 C**). H&E staining showed distinct necrotic regions in LV_342

tumors, not observed in LV_Control tumors (**Fig. 3 D**). This was also seen by fluorescence microscopy, since tumors from both cell types retained their dTomato expression. Necrotic regions (N) in LV_342 tumors showed no red fluorescence unlike areas with viable (V) cells. TUNEL assay showed significantly higher apoptotic cells in LV_342 tumors (**Fig. 3 E**). Our xenograft experiments thus indicate that miR-342-3p attenuates tumor growth.

Therapeutic miR-342-3p delivery inhibits HCC and improves survival of mice

To analyze therapeutic utility of miR-342-3p on liver tumor growth *in vivo*, we made an AAV-TTR-miR-342 vector (AAV-342), encoding miR-342-3p under the transcriptional control of liver specific transthyretin promoter, and a corresponding AAV-Control vector. Adult, tumor-bearing LT2/MYC mice, 8 weeks after c-MYC activation, were injected intravenously, with 5×10^{10} AAV8 viral particles, and sacrificed two weeks later (**Fig. 4 A**). AAV-Control mice developed large tumors and AAV-342 mice showed significant decrease in total tumor area (**Fig. 4 B-C**).

To determine whether the therapeutic potential of AAV-342 persists as tumors progress, mice were sacrificed at two additional timepoints, 3 weeks and 30 days (henceforth referred to as 4 weeks) following AAV-342 and AAV-Control treatment (**Fig. 4 A**). Significant decrease in total tumor area was also observed in AAV-342 mice sacrificed 3- and 4-weeks following treatment (**Fig. 4 B-C**).

We verified whether reduced tumor burden in AAV-342 mice can indeed be attributed to miR-342-3p, and whether increased miR-342-3p expression persists at later timepoints. We analyzed miR-342-3p expression in adjacent normal and tumor tissues from AAV-342 and AAV-Control mice livers, at the three timepoints. MiR-342-3p expression remained higher in AAV-342 mice compared to AAV-Control mice. Tumors from AAV-Control mice showed significantly lower expression of miR-342-3p compared to corresponding non-tumor liver tissue. However, AAV-342 mice tumors showed only slightly lower levels of miR-342-3p

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3 expression relative to adjacent non-tumor liver tissue. This pattern of expression was
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5 observed across all three timepoints following AAV treatment, albeit at varying levels (**Fig. 4**
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7 **D**). The AST/ALT ratio was significantly decreased in AAV-342 mice sera compared to AAV-
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9 Controls (**Supplementary Fig. S7 A**). We next determined if AAV-342 treatment *in vivo*
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11 affects cell proliferation and apoptosis. Ki67 immunohistochemical staining (**Fig. 4 E**) and
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13 TUNEL assay (**Fig. 4 F**) respectively revealed reduced proliferation and enhanced apoptosis
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15 in AAV-342 mice compared to AAV-Control mice, across all three timepoints (**Fig. 4 E-F**).
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20 We next determined the effect of AAV-342 on overall survival of tumor-bearing
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22 LT2/MYC mice. AAV-342 mice showed a median survival of 42.5 days compared to 28.5 days
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24 for AAV-Control mice, indicating a significant increase in overall survival (**Fig. 4 G**). Taken
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26 together, therapeutic miR-342-3p delivery attenuates tumor progression, even at 30 days
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28 following treatment, and prolongs survival in a mouse model of HCC.
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31 32 33 34 **Monocarboxylic acid transporter 1 (MCT1/ SLC16A1) is a direct target of miR-342-3p**

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37 To identify direct targets of miR-342-3p, we performed gene expression profiling on Huh7
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39 cells transfected with miR-342-3p mimic or scramble control. Following extensive *in silico*
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41 analyses, we narrowed down significantly downregulated genes that were predicted targets of
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43 miR-342-3p (Targetscan). To find a miR-342-3p target gene that would also be therapeutically
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45 relevant in HCC, we further narrowed down the >300 predicted targets to only those genes
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47 with a known role in cancer. This approach would enable us to understand the relevance and
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49 in-depth mechanism of miR-342-3p-mediated cancer inhibition, with the broader aim to be
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51 able to more efficiently therapeutically target HCC. We thus identified *MCT1* (*SLC16A1*)
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53 mRNA as a putative target of miR-342-3p in HCC (**Fig. 5 A, Supplementary Fig. S3**). GSEA
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55 analyses on a subset of genes with reported roles in glycolysis, TCA cycle metabolites,
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57 pyruvate metabolism and the solute carrier family, showed a strong negative correlation
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3 between miR-342-3p mimic- and scramble-treated cells (**Fig. 5 B**). *Mct1* was significantly
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5 downregulated in regressing tumors from LT2/MYC mice (**Fig. 5 C**). While *MCT1* was
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7 significantly downregulated in miR-342-3p mimic treated Huh7 cells, it was upregulated
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9 following miR-342-3p inhibitor treatment in LV_342 cells (**Fig. 5 D**), indicating that modulation
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11 of miR-342 expression inversely affects MCT1 levels. MCT1 protein expression in human
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13 hepatoma cell lines was higher than in normal primary human hepatocytes, and negatively
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15 correlated with miR-342-3p expression in these cell lines (**Supplementary Fig. S1 D and E**).
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17 Further, we found that MCT1 protein expression was downregulated in AAV-miR-342 mice
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19 across all three timepoints following AAV treatment (**Fig. 5 E**). Taken together, our findings
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21 point to regulation of MCT1 by miR-342-3p.
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27 To validate if *MCT1* is a *bona fide* miR-342-3p target, we investigated whether miR-
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29 342-3p binds to the 3'-UTR of *MCT1* in Huh7 cells, and whether disrupting the binding site
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31 would affect this interaction. Luciferase reporter assays on Huh7 cells co-transfected with
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33 either the pmirGLO vector, pmirGLO-MCT1-WT, or pmirGLO-MCT1-MUT, that we
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35 constructed, and the miR-342-3p mimic. Reduced luciferase activity was seen following co-
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37 transfection of pmirGLO-MCT1-WT with miR-342-3p mimic, but was not significantly altered
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39 compared to the control plasmid, when pmirGLO-MCT1-MUT was co-transfected with miR-
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41 342-3p mimic (**Fig. 5 F**). Our luciferase reporter assay thus confirmed that *MCT1* is a direct
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43 target of miR-342-3p.
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51 **Expression of miR-342-3p and MCT1 is inversely correlated in human HCC**

52 To determine whether miR-342-3p and MCT1 expression were inversely deregulated in
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54 human HCCs, tumor and matched non-tumor samples from 20 HCC patients were analyzed.
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56 While miR-342-3p expression was significantly lower in 75% of tumors compared to
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58 corresponding non-tumor tissues by qPCR (**Fig. 6 A, C, and Supplementary Fig. S4**), MCT1
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protein expression was higher in tumors (**Fig. 6 B, C**). Thus, miR-342-3p and MCT1 expression in human HCC biopsies are inversely correlated. Using the TCGA-LIHC database (8), we found downregulation of miR-342-3p by at least 2-fold in over 25% of primary tumor samples (n=96) (**Supplementary Fig. S5 A**). Significant negative correlation was observed between miR-342-3p and MCT1 in this dataset (**Supplementary Fig. S5 D**). Importantly, higher MCT1 was significantly associated with lower survival probability, whereas higher miR-342 was associated with higher survival probability (**Supplementary Fig. S5 C**). We next determined whether miR-342 downregulation was linked to c-Myc overexpression in this dataset. 42% samples with downregulated miR-342-3p showed varying levels of c-Myc upregulation (**Supplementary Fig. S5 B**). We found a negative correlation between miR-342 and MCT1 ($r = -0.55$) in samples with c-Myc > 3 (RNAseq HTSeq FPKM) (~5% of samples). Interestingly, selecting only samples (n=5) with both high c-Myc (> 3) and downregulated miR-342-3p, increased the inverse correlation between miR-342 and MCT1 ($r = -0.67$). Taken together, we found that miR-342-3p and MCT1 are indeed inversely deregulated in human HCCs (**Fig. 6, Supplementary Fig. S5 A-C**).

***MCT1* inhibition phenocopies miR-342-3p overexpression**

To ascertain whether miR-342-3p exerts its tumor suppressor function via MCT1 modulation, we treated Huh7 cells with the MCT1 inhibitor, AR-C141990 hydrochloride and analyzed cell proliferation (**Fig. 7 A**) and colony forming ability (**Fig. 7 B**), both of which were significantly decreased compared to untreated cells. We next inhibited miR-342-3p, with or without subsequent MCT1 inhibition, in LV_342 cells, and performed colony forming and wound healing assays. MiR-342-3p inhibition led to de-repression of MCT1, leading to increased colony formation and migration. Subsequent inhibition of increased MCT1 levels, via its inhibitor, led to inhibition of colony formation (**Fig. 7 C**) and slower migration (**Fig. 7 D**). These

findings indicate that miR-342-3p functions as a tumor suppressor, at least in part, via downregulation of MCT1.

MiR-342-3p overexpression affects the lactate transport function of MCT1

Inhibition of either monocarboxylate transporter, MCT1 or MCT4, may interrupt lactate transport. However, no significant difference in MCT4 levels in miR-342-3p mimic-treated cells (data not shown), ruled out MCT4 deregulation by miR-342-3p. To determine whether miR-342-3p-mediated MCT1 knockdown affects its role in lactate transport, we measured intracellular and extracellular lactate levels in LV_342 and LV_Control cells. We found higher extracellular lactate and significantly lower intracellular lactate levels (about 46% lower) in LV_342 cells (**Fig. 7 E**). Downregulation of MCT1 by miR-342-3p indeed impaired lactate transport. Further, following treatment with AR-C141990 hydrochloride, LV_342 cells continued to show lower intracellular lactate, since MCT1 was already downregulated by miR-342 (**Fig. 7 F**). MCT1 also transports pyruvate. Treatment with UK5099, an inhibitor of pyruvate transporter MPC, did not increase intracellular lactate accumulation in LV_Control cells, since lactate transport by MCT1 remained intact. Interestingly, significant increase in intracellular lactate was observed in LV_342 cells, since, in these cells, both MCT1-mediated bi-directional lactate transport into the cell and MPC-mediated pyruvate transport to the mitochondria were inhibited owing to miR-342 overexpression and UK5099 treatment, respectively (**Fig. 7 F**). We found no significant difference in LDHA protein expression in scramble or miR-342-3p inhibitor-treated LV_342 cells (data not shown). Thus, lower intracellular lactate in untreated LV_342 cells can indeed be largely attributed to MCT1 suppression by miR-342-3p, and is most likely not due to other effectors such as LDHA, MCT4, or MPC. Unexpectedly though, in spite of low intracellular lactate, significantly lower glucose uptake occurred in LV_342, as measured by two independent glucose uptake assays

(**Fig. 7 G and Supplementary Fig. S6 A**). However, no significant difference in expression of glucose transporter, GLUT1 was seen (data not shown). Also, LV_342 cells showed a marginal decrease in Extracellular acidification rate (ECAR) and a distinct decrease in Oxygen consumption rate (OCR), over time (**Supplementary Fig. S6 B**). Our results indicate overexpression of miR-342-3p, affects the lactate transport function of its target, MCT1.

Therapeutic miR-342-3p delivery inhibits HCC in an H-RAS-driven transgenic mouse model of HCC

To determine whether therapeutic efficacy of miR-342-2p, extends beyond the LT2/MYC mouse model, we used additional mouse models of HCC. Conditional doxycycline-regulatable HRAS-driven transgenic mouse model of HCC (LT2/RAS) (4), develop HCC following liver specific activation of H-RAS, following doxycycline withdrawal. Mice, off doxycycline for 4 weeks, were injected intravenously with AAV-342 or AAV-Control, and sacrificed two weeks after treatment (**Fig. 8A**). AAV-342 mice showed significant decrease in tumor development compared to AAV-Controls, as observed by an obvious difference in gross liver morphology (**Fig. 8 B**), and significantly lower liver /body weight ratios (**Fig. 8C, upper**). Significantly higher miR-342-3p expression in AAV-342 mice (**Fig. 8 C, lower**), indicated successful delivery and expression of AAV-342. Decreased proliferation and increased apoptosis, as determined by Ki67 staining and TUNEL assay, respectively (**Fig. 8 D**), and significant MCT1 downregulation (**Fig. 8 E**), occurred in AAV-342 mice livers.

To determine the effect of AAV-342 on survival, LT2/RAS mice off doxycycline for 5 weeks, were injected with either AAV-342 or AAV-Control. AAV-342 mice showed significant improvement in overall survival with a median survival of 39 days compared to 31 days in AAV-Controls (**Fig. 8 F**). We thus show therapeutic miR-342-3p delivery prolongs survival in two different mouse models of HCC.

AAV-342 suppresses tumor progression in a xenograft model of HCC

As a third HCC model, we injected 1×10^6 Huh-7 cells subcutaneously into flanks of female BALB/c Nu/Nu nude mice. Mice were subsequently injected intratumorally under anesthesia, with either AAV-342 or AAV-Control. AAV-342 injected tumors had lower tumor volumes (**Fig. 8 G**), tumor weights (**Fig. 8 H**), and showed a significantly higher expression of miR-342-3p (**Fig. 8 H**). Additionally, AAV-342 injected tumors had lower Ki67 and higher TUNEL positive cells compared to AAV-Control injected tumors (**Fig. 8 I**). The xenograft tumors also showed that AAV-342 treatment led to high miR-342-3p and downregulation of MCT1 expression (**Fig. 8 J**).

Taken together, our extensive *in vivo* analyses in three independent mouse models of HCC show that AAV-mediated delivery of miR-342-3p has a significant therapeutic effect on HCC tumor development *in vivo*, and highlights its therapeutic efficacy.

DISCUSSION

In our study, to identify the role miRNAs in tumor regression, we analyzed miRNA expression profiles of tumors and early regressing tumors from LT2/MYC mice, and independently identified miR-342-3p as a tumor suppressor miRNA, based initially on extensive *in silico* analyses of our array data and in-depth *in vitro* analyses with three other miRNA candidates. Notably, our study demonstrates, via both, in-depth *in vitro* and more importantly, extensive *in vivo* analyses in three independent mouse models of HCC, that therapeutic intervention with miR-342-3p has clinical implications in human HCCs. Potential clinical relevance for miR-342-3p is underlined by significant downregulation in human HCC biopsies we analyzed, compared to matched non-tumor counterparts. Additionally, miR-342 is downregulated in 25% primary liver tumor samples from the TCGA-LIHC dataset.

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3 Previous studies have also identified miR-342-3p in multiple cancers, including HCC (9-14).
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5 Gao, et. al, demonstrated the relevance of miR-342-3p as a prognostic marker in human HCC
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7 (11). MiR-342-3p was shown to be part of a 7-miRNA expression signature predicted to be
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9 involved in HBV-related HCC (12).
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12 We demonstrate a direct role for miR-342-3p in regulating tumor metabolism, in HCC,
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14 by targeting *MCT1*. *MCT1* is a widely expressed bi-directional transporter of
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16 monocarboxylates such as lactate and pyruvate (11) and is increased in cancer (12).
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18 Recently, *MCT1* was reported to be a miR-342-3p target in triple negative breast cancer (15),
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20 further highlighting our findings in HCC.
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24 MiR-342-3p may exert its tumor suppressor role by targeting genes other than *MCT1*.
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26 For example, miR-342-3p inhibits proliferation or induces apoptosis, by targeting genes
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28 associated with NF-kB (9) and Wnt/ β -catenin pathways (10, 13). MiR-342-3p decreases
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30 chemoresistance by cooperating with miR-205-5p to repress transcription factor E2F1 (16).
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34 We found that miR-342-3p affects lactate transport by targeting *MCT1* in HCC. LV_342
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36 cells showed increased extracellular lactate and decreased intracellular lactate, indicating
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38 inhibition of bi-directional lactate transport, and showed decreased glucose uptake. Treatment
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40 with *MCT1* inhibitor, SR13800, also showed marked decrease in glucose uptake in Burkitt's
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42 lymphoma (17). However, in contrast to our findings, they observed increased intracellular
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44 lactate accumulation. *MCT1* inhibition in triple negative breast cancer, led to higher
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46 extracellular and lower intracellular lactate, similar to our study (15), but showed a
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48 compensatory increase in glucose uptake. In another study, miR-342-3p modulated glycolysis
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50 by suppressing IGF-1R-mediated PI3K/AKT/GLUT1 signaling (14). Similar to our
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52 observations, increased miR-342-3p led to decreased glucose uptake, lactate levels, and
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54 ECAR. However, we also observed decreased OCR. Interestingly, miR-342-3p directly
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(MCT1) or indirectly (GLUT1 via IGF-1R) influences key modulators of glycolysis, at both ends of the process.

Oxygenated cancer cells may use lactate to fuel their metabolism with decreased glucose consumption. The contribution of circulating lactate to TCA cycle intermediates was shown to exceed that of glucose (18, 19). Therefore, it is possible that we do not see intracellular lactate accumulation in LV_342 cells which have inhibited MCT1, since lactate is possibly being reused for pyruvate, which in turn is used for glycolysis.

In summary, our study identifies miR-342-3p as a potent tumor suppressor miRNA with the ability to attenuate tumor development and positively influence tumor regression. We demonstrate a role for miR-342-3p in metabolic reprogramming of the tumor state by directly targeting MCT1, a key monocarboxylate transporter. We show that therapeutic administration of miR-342-3p leads to significant attenuation of liver tumors, *in vivo* and prolonged survival, thus highlighting the promising therapeutic potential of nucleic acid-based therapeutics in the treatment of HCC.

ABBREVIATIONS

4'-6-diamidino-2-phenylindole (Dapi), Fluorescence-activated cell sorting (FACS), hour (h).

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FIGURE LEGENDS

Fig. 1. MiRNA profiling reveals distinctly deregulated miRNAs during liver tumor regression and identifies miR-342-3p as a potential tumor suppressor. (A). Gross liver pictures and representative H&E stained sections depicting liver tumor and regressing tumor histology (magnification 40x). **(B)** Two-dimensional PCA plot showing distinct clustering of global miRNA expression profiles from tumor (T) and early regressing tumor (RT) groups (n=4 mice/group). **(C)** Heatmap showing hierarchical clustering of T and RT samples based on expression of selected potential tumor suppressor miRNAs. **(D)** qPCR-based quantification of relative expression of candidate tumor suppressor miRNAs in RT compared to T tissues. *, $P < 0.03$. Huh7 LV_miRNA stable cell lines were compared to LV_Control cells in **(E)** WST-1 cell proliferation assay, 48h post seeding. ***, $P < 0.0001$, **(F)** Soft agar colony forming assay showing relative colony numbers, two weeks post seeding. ***, $P < 1E-05$; **, $P < 0.0001$; *, $P < 0.01$, and in **(G)** Wound healing assays showing relative migration index (%), determined from the wound area measured with ImageJ. 0h and 24h post cell culture insert removal, are compared. The lower migration index indicates slower cell migration. ***, $P < 0.000082$; **, $P < 0.0027$; *, $P < 0.025$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

Fig. 2. In vitro loss of function analyses validates miR-342-3p as a potent tumor suppressor miRNA in HCC. (A) LV_342 cells show significantly decreased proliferation relative to LV_Control cells, with the WST-1 assay. MiR-342-3p inhibitor treatment rescues

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2
3 this inhibitory effect. 48h post transfection. ***, $P<0.0001$. **(B)** Representative images (20x
4 magnification) of immunofluorescence staining for Ki67 on LV_Control, LV_342, LV_342
5 scramble, and miR-342-3p inhibitor treated cells, 48h post transfection. Ki67 positive cells are
6 green and Dapi stained nuclei are blue. The corresponding graph shows an average % of
7 Ki67 positive cells from 15 fields/group. **, $P<0.002$, *, $P<0.02$. **(C)** Soft agar assay shows
8 decreased relative colony numbers in LV_342 compared to LV_Control cells and increased
9 relative colony numbers in miR-342-3p inhibitor treated LV_342 cells, at the two-week time
10 point. ***, $P<0.0001$. **(D)** Wound healing assay: At 48h post insert removal, the average, 0h
11 subtracted, Raw Integrated Density in the wound area for each group was quantified by
12 ImageJ. Higher raw integrated densities in the region of interest indicate increased cell
13 migration. **, $P<0.001$ *, $P<0.01$. **(E)** Representative dot plots (left) and graphs (right) with % of
14 Annexin V and dTomato positive cells from flow cytometry analyses of LV_Control scramble,
15 LV_342 scramble and miR-342-3p inhibitor treated cells, following APC- Annexin V staining.
16 **, $P<0.006$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

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39 **Fig. 3. MiR-342-3p attenuates development of xenograft tumors.** **(A)** Graph shows
40 average tumor volumes of all xenograft tumors developed from LV_342 cells compared to
41 LV_Control tumors. Mice/group: LV_Control: n=10; LV_342: n=7. *, $P<0.037$; **, $P<0.006$;
42 ***, $P<0.0003$. LV_342 tumors show **(B)** significantly lower average tumor weights. *, $P<0.05$.
43 and **(C)** continued miR-342-3p overexpression. ***, $P<0.0004$. **(D)** Histological comparison
44 between LV_342 tumors and LV_Control tumors shows extensive areas of necrotic tissue in
45 LV_342 tumors, not observed in the LV_Control group. This is seen with Hematoxylin and
46 Eosin (H&E) staining (above) and by immunofluorescence (IF) microscopy for dTomato and
47 Dapi. 'V' indicates regions with viable cells and 'N' indicates necrotic areas. **(E)** TUNEL
48 staining to detect apoptotic cells shows significantly higher TUNEL positive cells in LV_342
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3 compared to LV_Control xenograft tumors. Graph shows average % of TUNEL positive
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5 cells/high power field. Twenty high power fields/group were quantified. ***, $P < 0.0001$. Data
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7 are mean $\pm$ SEM; two-tailed Student's t-test.
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12 **Fig. 4. MiR-342-3p attenuates tumor growth and prolongs survival, *in vivo*. (A)**
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15 Schematic of *in vivo* AAV-miR-342-3p administration to study its effect on tumor development
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17 over three timepoints following AAV treatment, and on long-term survival of LT2/MYC mice.
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20 **(B)** Representative images of gross tumor-bearing LT2/MYC mice livers highlighting the tumor
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22 attenuating effect of AAV-342 compared to AAV-Control mice; 2, 3, and, 4 weeks (30 days)
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24 following AAV treatment. Mice/treatment group: 2 weeks: n=5, 3 weeks: n=4, 4 weeks: n=4.
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28 **(C)** Relative total tumor area of all tumors shows a significant decrease in AAV-342 mice, at
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30 all three timepoints post AAV treatment. ***, $P < 0.0004$, *, $P < 0.04$. **(D)** MiR-342-3p expression
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33 in tumors from AAV-342 and AAV-Control mice relative to corresponding adjacent non-tumor
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35 liver tissues, at all three timepoints post AAV treatment. *, $P < 0.028$, **, $P < 0.002$, ***,
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37 $P < 0.0004$. Representative images (20x magnification) of immunohistochemical staining for **(E)**

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39 Ki67 and **(F)** TUNEL from AAV-Control and AAV-342 mice. Graph shows relative average %
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41 Ki67 positive cells from 20 - 25 fields from AAV-342 mice relative to AAV-Control group, at all
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43 three timepoints following AAV treatment. **, $P < 0.007$, ***, $P < 3.7E-06$. The relative average
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45 number of TUNEL positive cells/high power field are shown for 40-50 fields per treatment
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47 group, at all three timepoints following AAV treatment. *, $P < 0.04$, ***, $P < 0.00001$. **(G)** Kaplan-

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49 Meier survival analysis shows a significant increase in overall survival of AAV-342 mice
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51 compared to AAV-Control mice (n=6 mice/group). Log-rank (Mantel-Cox) Test was used to
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53 compare the survival curves between the treatment groups. $P < 0.017$. Unless specified, data
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55 are mean $\pm$ SEM; two-tailed Student's t-test.
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Fig. 5. MCT1 is a *bona fide* direct target of miR-342-3p in HCC. (A) Flow chart depicting the *in-silico* approach taken to identify MCT1 as a potential miR-342-3p target. (B) GSEA enrichment plot depicting a strong negative correlation between selected genes including MCT1 in miR-342-3p mimic-treated Huh7 cells compared to scramble-treated cells. (C) Relative average *Mct1* mRNA expression in T and RT samples from LT2/MYC mice, showing significant knockdown in the RT group. **, $P < 0.005$. (D) Significant decrease of relative *MCT1* mRNA expression following miR-342-3p mimic treatment in LV_Control cells (left graph), is rescued by miR-342-3p inhibitor treatment in LV_342 cells (right graph). ***, $P < 0.0008$; **, $P < 0.002$. (E) MCT1 protein expression by western blot in AAV-Control and AAV-342 treated LT2/MYC mice tumors, at 2-, 3- and 4-weeks following AAV treatment, shows significant decrease in MCT1 expression in AAV-342 mice for all timepoints. 2 Weeks: **, $P < 0.008$, 3 Weeks: **, $P < 0.0025$, 4 Weeks **, $P < 0.003$. (F) Alignment of the putative binding site of miR-342 in *MCT1* 3'-UTR indicated with vertical lines. Mutated bases are shown in red. Luciferase assay shows reporter activity decreases relative to Control (pmirGLO empty vector and miR-342-3p mimic), when Wt-*MCT1* 3'-UTR is co-transfected with miR-342-3p mimic, and does not change significantly when Mut-*MCT1* 3'-UTR is used instead, indicating disrupted miRNA-target interaction. ***, $P < 0.0005$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

Fig. 6. MiR-342-3p and MCT1 are inversely correlated in human HCC. (A) Relative miR-342-3p expression from 20 HCC patients, shows significantly decreased miRNA expression in tumors compared to matched non-tumor liver tissues. *, $P < 0.05$. (B) Graph shows significantly increased relative MCT1 protein expression in tumors versus matched non-tumors from 18 HCC patients. A representative immunoblot with 6 matched tumor and non-tumor samples is shown. Densitometry-based relative protein quantification was carried out with ImageJ. ***, $P < 0.0001$. (C) Pie charts summarize proportion of HCC patients with downregulated miR-342-

3p and upregulated MCT1 protein expression, indicating an inverse expression pattern. Data are mean±SEM; two-tailed Student's t-test.

Fig. 7. MiR-342-3p overexpression affects the lactate transport function of MCT1.

LV_Control cells treated with 100uM of MCT1 inhibitor, AR-C141990 hydrochloride, show **(A)** decreased proliferation by WST1 assay relative to untreated cells, 48h post treatment. ***, P<0.0001 and **(B)** decreased average colony numbers in the soft agar colony forming assay. ***, P<0.0001. **(C)** Higher colony numbers in miR-342-3p inhibitor-treated LV_342 cells compared to scramble-treated cells, decreased following MCT1 inhibitor treatment. All soft agar assays were analysed 14 days after assay setup. ***, P<0.0001. **(D)** Representative images from wound healing assays in LV_342 scramble-treated cells, miR-342-3p inhibitor-treated cells, and miR-342-3p inhibitor-treated cells with subsequent MCT1 inhibitor treatment. Yellow boxes outline the starting wound area. Graph (right) shows migration index (%) determined from the wound area measured with ImageJ, 48h following insert removal compared to 0h. Lower migration index indicates slower cell migration. **, P<0.001; *, P<0.017; **(E)** Extracellular (***, P<0.0001) and intracellular (**, P<0.001) lactate levels in LV_Control and LV_342 cells, shows increased extracellular lactate and decreased intracellular lactate in LV_342. **(F)** Intracellular lactate levels in LV_342 cells relative to LV_Control cells in three groups: untreated, 100uM AR-C141990 hydrochloride treated, or 100uM UK5099 treated. **, P<0.001, *, P<0.04. **(G)** Glucose uptake assay shows significantly lower uptake in LV_342 cells. **, P<0.001. Unless specified, data are mean±SEM; two-tailed Student's t-test.

Fig. 8. MiR-342-3p attenuates tumor growth, *in vivo*, in two additional mouse models of HCC. (A) Schematic of AAV-miR-342-3p treatment to study its effect on tumor development

in LT2/RAS mice (n=4 mice/group) and Xenograft tumor mice (LV_Control: n=4, LV_342: n=5). **(B)** Representative gross images of tumor-bearing LT2/RAS mice livers highlighting the tumor attenuating effect of AAV-342, two weeks post AAV treatment. AAV-342 relative to AAV-Control mice show **(C)** significantly decreased total liver/body weight (upper). *, P<0.04, and, validated miR-342-3p overexpression (lower) in their tumors. **, P<0.001. **(D)** Representative images (20x magnification) following Ki67 staining (upper) and TUNEL assay (lower). Corresponding graphs show % Ki67 positive cells (30 fields/treatment group) ***, P<0.0001, and average numbers of TUNEL positive cells/high power field (40 fields/treatment group). ***, P<0.0001. AAV-342 mice compared to AAV-Control mice show **(E)** significant decrease in MCT1 protein expression by western blot *, P<0.04, and, **(F)** significant increase in overall survival, as determined by Kaplan-Meier survival analysis (n=5 mice/group). Log-rank (Mantel-Cox) Test compared survival curves between treatment groups. P<0.0018. **(G)** Representative images (left) and average tumor volumes (right) of xenograft tumors from nude mice injected with AAV-342 compared to AAV-Control, measured every alternate day and sacrificed one week following AAV treatment. *, P<0.02 **, P<0.004, ***, P<0.0001. **(H)** AAV-342 xenograft tumors show significantly lower tumor weights (left) *, P<0.01 and, overexpression of miR-342-3p (right). ***, P<0.001. **(I)** Representative images (20x magnification) of Ki67 staining (upper) and TUNEL assay (lower) from AAV-Control and AAV-342 injected xenograft tumors. Corresponding graphs show % Ki67 positive cells (30 fields/treatment group) ***, P<0.0003 and average number of TUNEL positive cells/high power field (30 fields/treatment group). *, P<0.01 **(J)** Western blot shows significantly decreased relative MCT1 protein expression in AAV-342 xenograft tumors compared to AAV-Controls. ***, P<0.0001. Unless specified, data are mean±SEM; two-tailed Student's t-test.

TITLE PAGE**MicroRNA-342-3p is a potent tumour suppressor in hepatocellular carcinoma**

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Authors contributions: A.B conceived the idea, designed the study, and provided the conceptual framework for the study. R-M.K, QH, and A.B performed all experiments and analyzed the data. L.vD and J.D helped with the cloning and the *in vitro* experiments. H-C.T helped with the xenograft experiments. Q.Y helped with animal experiments. O.O helped with the metabolic assays and luciferase reporter assays for the revised manuscript. AG provided us with the TRE-Myc, TRE-RAS, and LAP-tTA transgenic mouse models, which we bred and crossed to obtain the double transgenic LT2/MYC and LT2/RAS mice. A.B wrote the manuscript with the help of M.O, AD.S. and A.G. Y.X and R.Q helped with collection and analyses of human HCC tumor and matched non-tumor biopsies (China). A.B, M.P.M, M.O, and AD.S, provided conceptual evaluation of the project.

Abstract

Background & Aims: Hepatocellular carcinoma (HCC) is a cancer with multiple etiologies, and widespread prevalence. Largely refractory to current treatments, HCC is the fourth leading cause of cancer related deaths, world-wide. MicroRNAs (miRNAs) are important regulators in HCCs. We aimed to identify tumor suppressor miRNAs during tumor regression in a conditional c-MYC driven mouse model (LT2/MYC) of HCC, and to evaluate their therapeutic potential for HCC treatment. Methods: We performed miRNA expression profiling of developed and regressing LT2/MYC tumors, and in-depth *in vitro* gain- and loss-of-function analyses. Effect of adeno-associated virus vector (AAV)-mediated miR-342-3p treatment was evaluated in three HCC mouse models. Results: We identified miR-342-3p as a tumor suppressor miRNA in HCC, with increased expression in regressing tumors. Forced miR-342-3p expression in hepatoma cells showed significantly decreased cell proliferation, migration and colony formation. *In vivo* administration of AAV-miR-342-3p led to significant attenuation of tumor development and increased overall survival. We identified *Mct1* as a *bona fide* target of miR-342-3p in HCC. We show the tumor suppressor role of miR-342-3p is executed partly by modulating the lactate transport function of MCT1. Importantly, we find miR-342-3p downregulated in tumors from HCC patients compared to matched non-tumor tissues, inversely correlating with MCT1 expression. We observed similar findings in TCGA-LIHC data. Conclusion: In our study, we identified and validated miR-342-3p as a tumor suppressor miRNA, in HCC. We demonstrated its therapeutic efficacy in significantly attenuating tumor development, and prolonging survival, in different HCC mouse models. Identification of miR-342-3p as an effective tumor suppressor opens a therapeutic avenue for miRNA-mediated attenuation of HCC development.

Lay Summary

Hepatocellular carcinoma (HCC), the most common type of liver cancer, affects diverse populations and has global impact, being the fourth leading cause of cancer deaths, worldwide. HCC, which cannot be surgically removed, has no effective therapies for long-term survival. Thus, novel effective treatment options are urgently required. To understand the molecular basis of how tumors regress, we compared tumors and regressing liver tumors in mice. We show that small non-coding microRNA, miR-342-3p is a tumor suppressor in HCC. Expression of miR-342-3p is low in tumors and high in regressing tumors. When miR-342-3p is delivered to mouse livers with HCC, it can significantly slow down liver tumor development and improve survival. Our study highlights the promising therapeutic potential of miR-342-3p intervention in HCC.

INTRODUCTION

HCC is currently, a largely intractable disease. Apart from early surgical resection and liver transplantation, no effective cures currently exist. Patients with advanced unresectable HCC may receive tyrosine kinase inhibitors, Sorafenib, Lenvatinib, (1, 2), PD-L1 inhibitor Atezolizumab, or monoclonal antibody Bevacizumab, for first-line therapy, and Regorafenib for second-line treatment(3). Despite these advances, existing therapeutics are limited in their activity against HCC, and novel treatments need to be developed.

The property of hepatocytes to readily take up small nucleic acids opens novel therapeutic avenues against HCC. For example, small non-coding microRNAs (miRNAs), which play important regulatory roles in cancer development, show promising potential for miRNA-based therapies in HCC (4, 5).

Since *c-MYC* is a central gene in HCC, we have previously (4, 6) used conditional doxycycline-regulated LT2/MYC mice, to study HCC. Hepatocyte specific activation of *c-MYC* by removal of doxycycline-containing diet, gives rise to liver tumors (4), which readily regress when mice are re-fed a doxycycline-containing diet to represses MYC expression (6),(7). The LT2/MYC model thus permits us to study molecular events during tumor formation and the earliest stages of tumor regression.

To gain further insight into miRNA-based regulatory mechanisms in HCC regression, and explore new opportunities for therapeutic interventions, we performed miRNA expression profiling of tumors and regressing liver tumors from LT2/MYC mice. Following in-depth *in silico*, *in vitro* and xenograft analyses, we confirmed miR-342-3p as a *bona fide* tumor suppressor in HCC. AAV-mediated *in vivo* delivery of miR-342-3p, in three independent mouse models of HCC, led to significant attenuation of tumor progression and prolonged survival. We identified and validated Solute Carrier Family 16, Member 1 / Monocarboxylic Acid Transporter 1 (SLC16A1 / MCT1), as a direct target of miR-342-3p and showed that miR-

342-3p-mediated MCT1 knockdown affects its lactate transport function, in HCC. Our study highlights the therapeutic potential of miR-342-3p as a tumor suppressor miRNA in the treatment of HCC.

MATERIALS AND METHODS

Mouse models

All animal experiments were performed in accordance with ethical guidelines on animal care, directed by the Hannover medical school (MHH) and the approved animal protocol. Animals were housed in the MHH-Twincore animal facility and maintained with access to water and standard or doxycycline-containing food, *ad libitum*. We used adult LT2/MYC, LT2/RAS, and 8-12 weeks old BALB/cAnNRj-Foxn1 nu/nu mice. All experimental animals were closely monitored for their general health status.

MicroRNA expression profiling

We performed global microRNA expression profiling on liver tumors and regressing tumors from LT2/MYC mice. Total RNA was extracted from tissues (mirVana™ miRNA Isolation Kit, ThermoFisher Scientific). RNA was DNase-treated (Ambion DNase I (RNase-free), ThermoFisher Scientific) and sent to the MicroRNA Array Services, Exiqon for quality control (Bioanalyser 2100) followed by miRCURY™ LNA Array microRNA profiling.

Gene expression profiling

DNase-treated total RNA from Huh7 cells treated with miR-342 mimic or Scramble control was sent to the Genome Analytics core facility at the Helmholtz Center for Infection Research, Braunschweig, Germany, for global mRNA microarray profiling with the Agilent Whole Human Genome Oligo Microarrays (G4112A) platform.

In silico analyses

Cluster and TreeView were used to perform hierarchical clustering and generate heat maps.

Gene set enrichment analyses (GSEA) on subsets of energy metabolism genes was performed using mRNA array data from miR-342-3p mimic and scramble treated Huh7 cells.

Survival probability from the TCGA-LIHC dataset was analyzed by Xena Functional Genomics

Explorer resource (BioRxiv doi: <https://doi.org/10.1101/326470>). PCA plot showing separate

clustering of tumor (T) and early regressing tumor (RT) global miRNA expression profiles was

generated via Omicsoft, Oncoland array studio (trial version, Qiagen). Graphpad, Prism was

used to generate most of the graphs and the Kaplan-Meier analyses. Microsoft Excel or

Graphpad, Prism were used to calculate significance.

Adeno-associated virus serotype 8 (AAV8) production

293T cells were transfected with pDP8.ape (Plasmid Factory, Bielefeld, Germany) and pAAV-

Ttr-Control or pAAV-Ttr-mir-342 plasmids using the calcium phosphate AAV8 production

method established in our laboratory. Viral titers were determined by quantitative polymerase

chain reaction (qPCR) using primers targeting the Ttr promoter.

Statistical analyses

Significance was determined with two-tailed Student's t test for comparison of 2 groups. All p

<0.05 values were considered significant. The Kaplan-Meier analysis was used for survival

studies. Log-rank (Mantel-Cox) Test was used to compare survival curves between the

treatment groups. Error bars represent $\pm$ standard error of the mean (SEM).

Additional detailed methods are provided in Supplementary data and CTAT_table.

RESULTS

MiRNA profiling reveals distinctly deregulated miRNAs during liver tumor regression and identifies miR-342-3p as a tumor suppressor

The LT2/MYC mouse model enables controlled liver tumor development and regression by liver specific activation or repression of *c-MYC* via doxycycline (**Supplementary Fig. S1A**). Significantly reduced tumor burden was seen within three days after *c-MYC* inactivation (6) and histological differences were observed between tumors and early regressing tumors (**Fig. 1 A**). To identify miRNAs, which regulate tumor regression, we performed miRNA expression profiling and found significant differential regulation of global miRNA expression profiles from established tumors (T) and regressing tumors (RT), 72h after suppression of *c-MYC*. (**Fig. 1 B and Supplementary Fig. S1B**). Based on a >2-fold differential regulation and a $p < 0.05$, we identified (**Fig. 1 C**) and validated by quantitative PCR (qPCR), four miRNAs whose expression profiles indicated a potential function in tumor regression, with increased expression in the RT group - miR-342-3p, miR-4651, miR-136-5p, and miR-22-3p (**Fig. 1 D**). We performed gain-of-function studies to analyze their effects on characteristic hallmarks of cancer. Basal endogenous levels of these miRNAs in human hepatoma cell lines Huh7, HepG2, HepaRG and SNU182 compared to normal human hepatocytes, was first determined. Huh7 showed the lowest endogenous expression of all miRNA candidates (**Supplementary Fig. S1 C**) and was thus used to establish stable expression of individual miRNAs with respective lentiviral vectors (LV-miR) (representative example in **Supplementary Fig. S2 A**). A corresponding control Huh7 cell line (LV_Control), expressed dTomato, but no miRNA. We found only Huh7 LV_342 cells, stably expressing miR-342-3p and a dTomato reporter, showed a consistent decrease in proliferation, 48h post cell seeding, with the WST-1 cell proliferation assay (**Fig. 1 E**), and, most robustly and reproducibly suppressed both colony

forming ability in soft agar assays (**Fig. 1 F**), and migration of cells in wound healing assays (**Fig. 1 G**), compared to LV_Control cells. Our miRNA expression profiling, validation in mouse liver tumors, and *in vitro* functional analyses, strongly support a tumor suppressor role for miR-342-3p, which was thus selected for further study.

MiR-342-3p suppresses growth of HCC cells, *in vitro* and in a xenograft tumor model

To determine whether growth inhibitory effects in LV_342 cells indeed resulted from specific gain of miR-342-3p, we inhibited miR-342-3p by transfecting LV_342 cells with a miR-342-3p inhibitor. MiR-342-3p inhibition was confirmed by qPCR (**Supplementary Fig. S2 B**). We found that miR-342-3p inhibitor treatment rescued inhibitory effects of miR-342-3p seen in comparison to LV_Control scramble-treated cells (**Fig. 2 A-C**). MiR-342-3p inhibition led to significant increase in cell proliferation with the WST-1 assay (**Fig. 2 A**), a higher number of proliferation marker, Ki67-positive cells by immunofluorescence staining (**Fig. 2 B**), increased number of colonies by soft agar colony forming assay (**Fig. 2 C**), and increased cell migration by wound healing assay (**Fig. 2 D, Supplementary Fig. S2 C**). Annexin V staining followed by FACS, showed significant increase in apoptosis in LV_342 Scr cells compared to LV_Control Scr cells, which significantly decreased following miR-342-3p inhibition (**Fig. 2 E**), indicating rescue of the pro-apoptotic effect of miR-342-3p. Hence, our *in vitro* gain- and loss-of-function experiments clearly indicate that miR-342-3p is a *bona fide* tumor suppressor miRNA.

To investigate whether miR-342-3p regulates tumorigenicity in a xenograft tumor model, we injected 1×10^6 LV_342 or LV_Control cells subcutaneously into flanks of female BALB/c Nu/Nu nude mice. Significant attenuation of tumor development and decreased tumor volumes and weights, were seen in LV_342 tumors (**Fig. 3 A-B**), which still showed high miR-342-3p expression (**Fig. 3 C**). H&E staining showed distinct necrotic regions in LV_342

tumors, not observed in LV_Control tumors (**Fig. 3 D**). This was also seen by fluorescence microscopy, since tumors from both cell types retained their dTomato expression. Necrotic regions (N) in LV_342 tumors showed no red fluorescence unlike areas with viable (V) cells. TUNEL assay showed significantly higher apoptotic cells in LV_342 tumors (**Fig. 3 E**). Our xenograft experiments thus indicate that miR-342-3p attenuates tumor growth.

Therapeutic miR-342-3p delivery inhibits HCC and improves survival of mice

To analyze therapeutic utility of miR-342-3p on liver tumor growth *in vivo*, we made an AAV-TTR-miR-342 vector (AAV-342), encoding miR-342-3p under the transcriptional control of liver specific transthyretin promoter, and a corresponding AAV-Control vector. Adult, tumor-bearing LT2/MYC mice, 8 weeks after c-MYC activation, were injected intravenously, with 5×10^{10} AAV8 viral particles, and sacrificed two weeks later (**Fig. 4 A**). AAV-Control mice developed large tumors and AAV-342 mice showed significant decrease in total tumor area (**Fig. 4 B-C**).

To determine whether the therapeutic potential of AAV-342 persists as tumors progress, mice were sacrificed at two additional timepoints, 3 weeks and 30 days (henceforth referred to as 4 weeks) following AAV-342 and AAV-Control treatment (**Fig. 4 A**). Significant decrease in total tumor area was also observed in AAV-342 mice sacrificed 3- and 4-weeks following treatment (**Fig. 4 B-C**).

We verified whether reduced tumor burden in AAV-342 mice can indeed be attributed to miR-342-3p, and whether increased miR-342-3p expression persists at later timepoints. We analyzed miR-342-3p expression in adjacent normal and tumor tissues from AAV-342 and AAV-Control mice livers, at the three timepoints. MiR-342-3p expression remained higher in AAV-342 mice compared to AAV-Control mice. Tumors from AAV-Control mice showed significantly lower expression of miR-342-3p compared to corresponding non-tumor liver tissue. However, AAV-342 mice tumors showed only slightly lower levels of miR-342-3p

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3 expression relative to adjacent non-tumor liver tissue. This pattern of expression was
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5 observed across all three timepoints following AAV treatment, albeit at varying levels (**Fig. 4**
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7 **D**). The AST/ALT ratio was significantly decreased in AAV-342 mice sera compared to AAV-
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9 Controls (**Supplementary Fig. S7 A**). We next determined if AAV-342 treatment *in vivo*
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11 affects cell proliferation and apoptosis. Ki67 immunohistochemical staining (**Fig. 4 E**) and
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13 TUNEL assay (**Fig. 4 F**) respectively revealed reduced proliferation and enhanced apoptosis
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15 in AAV-342 mice compared to AAV-Control mice, across all three timepoints (**Fig. 4 E-F**).
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20 We next determined the effect of AAV-342 on overall survival of tumor-bearing
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22 LT2/MYC mice. AAV-342 mice showed a median survival of 42.5 days compared to 28.5 days
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24 for AAV-Control mice, indicating a significant increase in overall survival (**Fig. 4 G**). Taken
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26 together, therapeutic miR-342-3p delivery attenuates tumor progression, even at 30 days
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28 following treatment, and prolongs survival in a mouse model of HCC.
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31 32 33 34 **Monocarboxylic acid transporter 1 (MCT1/ SLC16A1) is a direct target of miR-342-3p**

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37 To identify direct targets of miR-342-3p, we performed gene expression profiling on Huh7
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39 cells transfected with miR-342-3p mimic or scramble control. Following extensive *in silico*
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41 analyses, we narrowed down significantly downregulated genes that were predicted targets of
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43 miR-342-3p (Targetscan). To find a miR-342-3p target gene that would also be therapeutically
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45 relevant in HCC, we further narrowed down the >300 predicted targets to only those genes
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47 with a known role in cancer. This approach would enable us to understand the relevance and
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49 in-depth mechanism of miR-342-3p-mediated cancer inhibition, with the broader aim to be
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51 able to more efficiently therapeutically target HCC. We thus identified *MCT1* (*SLC16A1*)
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53 mRNA as a putative target of miR-342-3p in HCC (**Fig. 5 A, Supplementary Fig. S3**). GSEA
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55 analyses on a subset of genes with reported roles in glycolysis, TCA cycle metabolites,
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57 pyruvate metabolism and the solute carrier family, showed a strong negative correlation
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3 between miR-342-3p mimic- and scramble-treated cells (**Fig. 5 B**). *Mct1* was significantly
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5 downregulated in regressing tumors from LT2/MYC mice (**Fig. 5 C**). While *MCT1* was
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7 significantly downregulated in miR-342-3p mimic treated Huh7 cells, it was upregulated
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9 following miR-342-3p inhibitor treatment in LV_342 cells (**Fig. 5 D**), indicating that modulation
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11 of miR-342 expression inversely affects MCT1 levels. MCT1 protein expression in human
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13 hepatoma cell lines was higher than in normal primary human hepatocytes, and negatively
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15 correlated with miR-342-3p expression in these cell lines (**Supplementary Fig. S1 D and E**).
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17 Further, we found that MCT1 protein expression was downregulated in AAV-miR-342 mice
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19 across all three timepoints following AAV treatment (**Fig. 5 E**). Taken together, our findings
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21 point to regulation of MCT1 by miR-342-3p.
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27 To validate if *MCT1* is a *bona fide* miR-342-3p target, we investigated whether miR-
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29 342-3p binds to the 3'-UTR of *MCT1* in Huh7 cells, and whether disrupting the binding site
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31 would affect this interaction. Luciferase reporter assays on Huh7 cells co-transfected with
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33 either the pmirGLO vector, pmirGLO-MCT1-WT, or pmirGLO-MCT1-MUT, that we
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35 constructed, and the miR-342-3p mimic. Reduced luciferase activity was seen following co-
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37 transfection of pmirGLO-MCT1-WT with miR-342-3p mimic, but was not significantly altered
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39 compared to the control plasmid, when pmirGLO-MCT1-MUT was co-transfected with miR-
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41 342-3p mimic (**Fig. 5 F**). Our luciferase reporter assay thus confirmed that *MCT1* is a direct
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43 target of miR-342-3p.
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51 **Expression of miR-342-3p and MCT1 is inversely correlated in human HCC**

52 To determine whether miR-342-3p and MCT1 expression were inversely deregulated in
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54 human HCCs, tumor and matched non-tumor samples from 20 HCC patients were analyzed.
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56 While miR-342-3p expression was significantly lower in 75% of tumors compared to
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58 corresponding non-tumor tissues by qPCR (**Fig. 6 A, C, and Supplementary Fig. S4**), MCT1
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protein expression was higher in tumors (**Fig. 6 B, C**). Thus, miR-342-3p and MCT1 expression in human HCC biopsies are inversely correlated. Using the TCGA-LIHC database (8), we found downregulation of miR-342-3p by at least 2-fold in over 25% of primary tumor samples (n=96) (**Supplementary Fig. S5 A**). Significant negative correlation was observed between miR-342-3p and MCT1 in this dataset (**Supplementary Fig. S5 D**). Importantly, higher MCT1 was significantly associated with lower survival probability, whereas higher miR-342 was associated with higher survival probability (**Supplementary Fig. S5 C**). We next determined whether miR-342 downregulation was linked to c-Myc overexpression in this dataset. 42% samples with downregulated miR-342-3p showed varying levels of c-Myc upregulation (**Supplementary Fig. S5 B**). We found a negative correlation between miR-342 and MCT1 ($r = -0.55$) in samples with c-Myc > 3 (RNAseq HTSeq FPKM) (~5% of samples). Interestingly, selecting only samples (n=5) with both high c-Myc (> 3) and downregulated miR-342-3p, increased the inverse correlation between miR-342 and MCT1 ($r = -0.67$). Taken together, we found that miR-342-3p and MCT1 are indeed inversely deregulated in human HCCs (**Fig. 6, Supplementary Fig. S5 A-C**).

***MCT1* inhibition phenocopies miR-342-3p overexpression**

To ascertain whether miR-342-3p exerts its tumor suppressor function via MCT1 modulation, we treated Huh7 cells with the MCT1 inhibitor, AR-C141990 hydrochloride and analyzed cell proliferation (**Fig. 7 A**) and colony forming ability (**Fig. 7 B**), both of which were significantly decreased compared to untreated cells. We next inhibited miR-342-3p, with or without subsequent MCT1 inhibition, in LV_342 cells, and performed colony forming and wound healing assays. MiR-342-3p inhibition led to de-repression of MCT1, leading to increased colony formation and migration. Subsequent inhibition of increased MCT1 levels, via its inhibitor, led to inhibition of colony formation (**Fig. 7 C**) and slower migration (**Fig. 7 D**). These

findings indicate that miR-342-3p functions as a tumor suppressor, at least in part, via downregulation of MCT1.

MiR-342-3p overexpression affects the lactate transport function of MCT1

Inhibition of either monocarboxylate transporter, MCT1 or MCT4, may interrupt lactate transport. However, no significant difference in MCT4 levels in miR-342-3p mimic-treated cells (data not shown), ruled out MCT4 deregulation by miR-342-3p. To determine whether miR-342-3p-mediated MCT1 knockdown affects its role in lactate transport, we measured intracellular and extracellular lactate levels in LV_342 and LV_Control cells. We found higher extracellular lactate and significantly lower intracellular lactate levels (about 46% lower) in LV_342 cells (**Fig. 7 E**). Downregulation of MCT1 by miR-342-3p indeed impaired lactate transport. Further, following treatment with AR-C141990 hydrochloride, LV_342 cells continued to show lower intracellular lactate, since MCT1 was already downregulated by miR-342 (**Fig. 7 F**). MCT1 also transports pyruvate. Treatment with UK5099, an inhibitor of pyruvate transporter MPC, did not increase intracellular lactate accumulation in LV_Control cells, since lactate transport by MCT1 remained intact. Interestingly, significant increase in intracellular lactate was observed in LV_342 cells, since, in these cells, both MCT1-mediated bi-directional lactate transport into the cell and MPC-mediated pyruvate transport to the mitochondria were inhibited owing to miR-342 overexpression and UK5099 treatment, respectively (**Fig. 7 F**). We found no significant difference in LDHA protein expression in scramble or miR-342-3p inhibitor-treated LV_342 cells (data not shown). Thus, lower intracellular lactate in untreated LV_342 cells can indeed be largely attributed to MCT1 suppression by miR-342-3p, and is most likely not due to other effectors such as LDHA, MCT4, or MPC. Unexpectedly though, in spite of low intracellular lactate, significantly lower glucose uptake occurred in LV_342, as measured by two independent glucose uptake assays

(**Fig. 7 G and Supplementary Fig. S6 A**). However, no significant difference in expression of glucose transporter, GLUT1 was seen (data not shown). Also, LV_342 cells showed a marginal decrease in Extracellular acidification rate (ECAR) and a distinct decrease in Oxygen consumption rate (OCR), over time (**Supplementary Fig. S6 B**). Our results indicate overexpression of miR-342-3p, affects the lactate transport function of its target, MCT1.

Therapeutic miR-342-3p delivery inhibits HCC in an H-RAS-driven transgenic mouse model of HCC

To determine whether therapeutic efficacy of miR-342-2p, extends beyond the LT2/MYC mouse model, we used additional mouse models of HCC. Conditional doxycycline-regulatable HRAS-driven transgenic mouse model of HCC (LT2/RAS) (4), develop HCC following liver specific activation of H-RAS, following doxycycline withdrawal. Mice, off doxycycline for 4 weeks, were injected intravenously with AAV-342 or AAV-Control, and sacrificed two weeks after treatment (**Fig. 8A**). AAV-342 mice showed significant decrease in tumor development compared to AAV-Controls, as observed by an obvious difference in gross liver morphology (**Fig. 8 B**), and significantly lower liver /body weight ratios (**Fig. 8C, upper**). Significantly higher miR-342-3p expression in AAV-342 mice (**Fig. 8 C, lower**), indicated successful delivery and expression of AAV-342. Decreased proliferation and increased apoptosis, as determined by Ki67 staining and TUNEL assay, respectively (**Fig. 8 D**), and significant MCT1 downregulation (**Fig. 8 E**), occurred in AAV-342 mice livers.

To determine the effect of AAV-342 on survival, LT2/RAS mice off doxycycline for 5 weeks, were injected with either AAV-342 or AAV-Control. AAV-342 mice showed significant improvement in overall survival with a median survival of 39 days compared to 31 days in AAV-Controls (**Fig. 8 F**). We thus show therapeutic miR-342-3p delivery prolongs survival in two different mouse models of HCC.

AAV-342 suppresses tumor progression in a xenograft model of HCC

As a third HCC model, we injected 1×10^6 Huh-7 cells subcutaneously into flanks of female BALB/c Nu/Nu nude mice. Mice were subsequently injected intratumorally under anesthesia, with either AAV-342 or AAV-Control. AAV-342 injected tumors had lower tumor volumes (**Fig. 8 G**), tumor weights (**Fig. 8 H**), and showed a significantly higher expression of miR-342-3p (**Fig. 8 H**). Additionally, AAV-342 injected tumors had lower Ki67 and higher TUNEL positive cells compared to AAV-Control injected tumors (**Fig. 8 I**). The xenograft tumors also showed that AAV-342 treatment led to high miR-342-3p and downregulation of MCT1 expression (**Fig. 8 J**).

Taken together, our extensive *in vivo* analyses in three independent mouse models of HCC show that AAV-mediated delivery of miR-342-3p has a significant therapeutic effect on HCC tumor development *in vivo*, and highlights its therapeutic efficacy.

DISCUSSION

In our study, to identify the role miRNAs in tumor regression, we analyzed miRNA expression profiles of tumors and early regressing tumors from LT2/MYC mice, and independently identified miR-342-3p as a tumor suppressor miRNA, based initially on extensive *in silico* analyses of our array data and in-depth *in vitro* analyses with three other miRNA candidates. Notably, our study demonstrates, via both, in-depth *in vitro* and more importantly, extensive *in vivo* analyses in three independent mouse models of HCC, that therapeutic intervention with miR-342-3p has clinical implications in human HCCs. Potential clinical relevance for miR-342-3p is underlined by significant downregulation in human HCC biopsies we analyzed, compared to matched non-tumor counterparts. Additionally, miR-342 is downregulated in 25% primary liver tumor samples from the TCGA-LIHC dataset.

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3 Previous studies have also identified miR-342-3p in multiple cancers, including HCC (9-14).
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5 Gao, et. al, demonstrated the relevance of miR-342-3p as a prognostic marker in human HCC
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7 (11). MiR-342-3p was shown to be part of a 7-miRNA expression signature predicted to be
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9 involved in HBV-related HCC (12).
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12 We demonstrate a direct role for miR-342-3p in regulating tumor metabolism, in HCC,
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14 by targeting *MCT1*. *MCT1* is a widely expressed bi-directional transporter of
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16 monocarboxylates such as lactate and pyruvate (11) and is increased in cancer (12).
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18 Recently, *MCT1* was reported to be a miR-342-3p target in triple negative breast cancer (15),
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20 further highlighting our findings in HCC.
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24 MiR-342-3p may exert its tumor suppressor role by targeting genes other than *MCT1*.
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26 For example, miR-342-3p inhibits proliferation or induces apoptosis, by targeting genes
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28 associated with NF-kB (9) and Wnt/ β -catenin pathways (10, 13). MiR-342-3p decreases
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30 chemoresistance by cooperating with miR-205-5p to repress transcription factor E2F1 (16).
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34 We found that miR-342-3p affects lactate transport by targeting *MCT1* in HCC. LV_342
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36 cells showed increased extracellular lactate and decreased intracellular lactate, indicating
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38 inhibition of bi-directional lactate transport, and showed decreased glucose uptake. Treatment
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40 with *MCT1* inhibitor, SR13800, also showed marked decrease in glucose uptake in Burkitt's
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42 lymphoma (17). However, in contrast to our findings, they observed increased intracellular
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44 lactate accumulation. *MCT1* inhibition in triple negative breast cancer, led to higher
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46 extracellular and lower intracellular lactate, similar to our study (15), but showed a
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48 compensatory increase in glucose uptake. In another study, miR-342-3p modulated glycolysis
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50 by suppressing IGF-1R-mediated PI3K/AKT/GLUT1 signaling (14). Similar to our
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52 observations, increased miR-342-3p led to decreased glucose uptake, lactate levels, and
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54 ECAR. However, we also observed decreased OCR. Interestingly, miR-342-3p directly
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(MCT1) or indirectly (GLUT1 via IGF-1R) influences key modulators of glycolysis, at both ends of the process.

Oxygenated cancer cells may use lactate to fuel their metabolism with decreased glucose consumption. The contribution of circulating lactate to TCA cycle intermediates was shown to exceed that of glucose (18, 19). Therefore, it is possible that we do not see intracellular lactate accumulation in LV_342 cells which have inhibited MCT1, since lactate is possibly being reused for pyruvate, which in turn is used for glycolysis.

In summary, our study identifies miR-342-3p as a potent tumor suppressor miRNA with the ability to attenuate tumor development and positively influence tumor regression. We demonstrate a role for miR-342-3p in metabolic reprogramming of the tumor state by directly targeting MCT1, a key monocarboxylate transporter. We show that therapeutic administration of miR-342-3p leads to significant attenuation of liver tumors, *in vivo* and prolonged survival, thus highlighting the promising therapeutic potential of nucleic acid-based therapeutics in the treatment of HCC.

ABBREVIATIONS

4'-6-diamidino-2-phenylindole (Dapi), Fluorescence-activated cell sorting (FACS), hour (h).

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Author names in bold designate shared co-first authorship.

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FIGURE LEGENDS

Fig. 1. MiRNA profiling reveals distinctly deregulated miRNAs during liver tumor regression and identifies miR-342-3p as a potential tumor suppressor. (A). Gross liver pictures and representative H&E stained sections depicting liver tumor and regressing tumor histology (magnification 40x). **(B)** Two-dimensional PCA plot showing distinct clustering of global miRNA expression profiles from tumor (T) and early regressing tumor (RT) groups (n=4 mice/group). **(C)** Heatmap showing hierarchical clustering of T and RT samples based on expression of selected potential tumor suppressor miRNAs. **(D)** qPCR-based quantification of relative expression of candidate tumor suppressor miRNAs in RT compared to T tissues. *, $P < 0.03$. Huh7 LV_miRNA stable cell lines were compared to LV_Control cells in **(E)** WST-1 cell proliferation assay, 48h post seeding. ***, $P < 0.0001$, **(F)** Soft agar colony forming assay showing relative colony numbers, two weeks post seeding. ***, $P < 1E-05$; **, $P < 0.0001$; *, $P < 0.01$, and in **(G)** Wound healing assays showing relative migration index (%), determined from the wound area measured with ImageJ. 0h and 24h post cell culture insert removal, are compared. The lower migration index indicates slower cell migration. ***, $P < 0.000082$; **, $P < 0.0027$; *, $P < 0.025$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

Fig. 2. *In vitro* loss of function analyses validates miR-342-3p as a potent tumor suppressor miRNA in HCC. (A) LV_342 cells show significantly decreased proliferation relative to LV_Control cells, with the WST-1 assay. MiR-342-3p inhibitor treatment rescues

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3 this inhibitory effect. 48h post transfection. ***, $P < 0.0001$. **(B)** Representative images (20x
4 magnification) of immunofluorescence staining for Ki67 on LV_Control, LV_342, LV_342
5 scramble, and miR-342-3p inhibitor treated cells, 48h post transfection. Ki67 positive cells are
6 green and Dapi stained nuclei are blue. The corresponding graph shows an average % of
7 Ki67 positive cells from 15 fields/group. **, $P < 0.002$, *, $P < 0.02$. **(C)** Soft agar assay shows
8 decreased relative colony numbers in LV_342 compared to LV_Control cells and increased
9 relative colony numbers in miR-342-3p inhibitor treated LV_342 cells, at the two-week time
10 point. ***, $P < 0.0001$. **(D)** Wound healing assay: At 48h post insert removal, the average, 0h
11 subtracted, Raw Integrated Density in the wound area for each group was quantified by
12 ImageJ. Higher raw integrated densities in the region of interest indicate increased cell
13 migration. **, $P < 0.001$ *, $P < 0.01$. **(E)** Representative dot plots (left) and graphs (right) with % of
14 Annexin V and dTomato positive cells from flow cytometry analyses of LV_Control scramble,
15 LV_342 scramble and miR-342-3p inhibitor treated cells, following APC- Annexin V staining.
16 **, $P < 0.006$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

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39 **Fig. 3. MiR-342-3p attenuates development of xenograft tumors.** **(A)** Graph shows
40 average tumor volumes of all xenograft tumors developed from LV_342 cells compared to
41 LV_Control tumors. Mice/group: LV_Control: n=10; LV_342: n=7. *, $P < 0.037$; **, $P < 0.006$;
42 ***, $P < 0.0003$. LV_342 tumors show **(B)** significantly lower average tumor weights. *, $P < 0.05$.
43 and **(C)** continued miR-342-3p overexpression. ***, $P < 0.0004$. **(D)** Histological comparison
44 between LV_342 tumors and LV_Control tumors shows extensive areas of necrotic tissue in
45 LV_342 tumors, not observed in the LV_Control group. This is seen with Hematoxylin and
46 Eosin (H&E) staining (above) and by immunofluorescence (IF) microscopy for dTomato and
47 Dapi. 'V' indicates regions with viable cells and 'N' indicates necrotic areas. **(E)** TUNEL
48 staining to detect apoptotic cells shows significantly higher TUNEL positive cells in LV_342
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3 compared to LV_Control xenograft tumors. Graph shows average % of TUNEL positive
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5 cells/high power field. Twenty high power fields/group were quantified. ***, $P < 0.0001$. Data
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7 are mean $\pm$ SEM; two-tailed Student's t-test.
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12 **Fig. 4. MiR-342-3p attenuates tumor growth and prolongs survival, *in vivo*. (A)**
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15 Schematic of *in vivo* AAV-miR-342-3p administration to study its effect on tumor development
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17 over three timepoints following AAV treatment, and on long-term survival of LT2/MYC mice.
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20 **(B)** Representative images of gross tumor-bearing LT2/MYC mice livers highlighting the tumor
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22 attenuating effect of AAV-342 compared to AAV-Control mice; 2, 3, and, 4 weeks (30 days)
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24 following AAV treatment. Mice/treatment group: 2 weeks: n=5, 3 weeks: n=4, 4 weeks: n=4.
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28 **(C)** Relative total tumor area of all tumors shows a significant decrease in AAV-342 mice, at
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30 all three timepoints post AAV treatment. ***, $P < 0.0004$, *, $P < 0.04$. **(D)** MiR-342-3p expression
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33 in tumors from AAV-342 and AAV-Control mice relative to corresponding adjacent non-tumor
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35 liver tissues, at all three timepoints post AAV treatment. *, $P < 0.028$, **, $P < 0.002$, ***,
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37 $P < 0.0004$. Representative images (20x magnification) of immunohistochemical staining for **(E)**

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39 Ki67 and **(F)** TUNEL from AAV-Control and AAV-342 mice. Graph shows relative average %
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41 Ki67 positive cells from 20 - 25 fields from AAV-342 mice relative to AAV-Control group, at all
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43 three timepoints following AAV treatment. **, $P < 0.007$, ***, $P < 3.7E-06$. The relative average
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45 number of TUNEL positive cells/high power field are shown for 40-50 fields per treatment
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47 group, at all three timepoints following AAV treatment. *, $P < 0.04$, ***, $P < 0.00001$. **(G)** Kaplan-

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49 Meier survival analysis shows a significant increase in overall survival of AAV-342 mice
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51 compared to AAV-Control mice (n=6 mice/group). Log-rank (Mantel-Cox) Test was used to
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53 compare the survival curves between the treatment groups. $P < 0.017$. Unless specified, data
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55 are mean $\pm$ SEM; two-tailed Student's t-test.
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Fig. 5. MCT1 is a *bona fide* direct target of miR-342-3p in HCC. (A) Flow chart depicting the *in-silico* approach taken to identify MCT1 as a potential miR-342-3p target. (B) GSEA enrichment plot depicting a strong negative correlation between selected genes including MCT1 in miR-342-3p mimic-treated Huh7 cells compared to scramble-treated cells. (C) Relative average *Mct1* mRNA expression in T and RT samples from LT2/MYC mice, showing significant knockdown in the RT group. **, $P < 0.005$. (D) Significant decrease of relative *MCT1* mRNA expression following miR-342-3p mimic treatment in LV_Control cells (left graph), is rescued by miR-342-3p inhibitor treatment in LV_342 cells (right graph). ***, $P < 0.0008$; **, $P < 0.002$. (E) MCT1 protein expression by western blot in AAV-Control and AAV-342 treated LT2/MYC mice tumors, at 2-, 3- and 4-weeks following AAV treatment, shows significant decrease in MCT1 expression in AAV-342 mice for all timepoints. 2 Weeks: **, $P < 0.008$, 3 Weeks: **, $P < 0.0025$, 4 Weeks **, $P < 0.003$. (F) Alignment of the putative binding site of miR-342 in *MCT1* 3'-UTR indicated with vertical lines. Mutated bases are shown in red. Luciferase assay shows reporter activity decreases relative to Control (pmirGLO empty vector and miR-342-3p mimic), when Wt-*MCT1* 3'-UTR is co-transfected with miR-342-3p mimic, and does not change significantly when Mut-*MCT1* 3'-UTR is used instead, indicating disrupted miRNA-target interaction. ***, $P < 0.0005$. Data are mean $\pm$ SEM; two-tailed Student's t-test.

Fig. 6. MiR-342-3p and MCT1 are inversely correlated in human HCC. (A) Relative miR-342-3p expression from 20 HCC patients, shows significantly decreased miRNA expression in tumors compared to matched non-tumor liver tissues. *, $P < 0.05$. (B) Graph shows significantly increased relative MCT1 protein expression in tumors versus matched non-tumors from 18 HCC patients. A representative immunoblot with 6 matched tumor and non-tumor samples is shown. Densitometry-based relative protein quantification was carried out with ImageJ. ***, $P < 0.0001$. (C) Pie charts summarize proportion of HCC patients with downregulated miR-342-

3p and upregulated MCT1 protein expression, indicating an inverse expression pattern. Data are mean±SEM; two-tailed Student's t-test.

Fig. 7. MiR-342-3p overexpression affects the lactate transport function of MCT1.

LV_Control cells treated with 100uM of MCT1 inhibitor, AR-C141990 hydrochloride, show **(A)** decreased proliferation by WST1 assay relative to untreated cells, 48h post treatment. ***, P<0.0001 and **(B)** decreased average colony numbers in the soft agar colony forming assay. ***, P<0.0001. **(C)** Higher colony numbers in miR-342-3p inhibitor-treated LV_342 cells compared to scramble-treated cells, decreased following MCT1 inhibitor treatment. All soft agar assays were analysed 14 days after assay setup. ***, P<0.0001. **(D)** Representative images from wound healing assays in LV_342 scramble-treated cells, miR-342-3p inhibitor-treated cells, and miR-342-3p inhibitor-treated cells with subsequent MCT1 inhibitor treatment. Yellow boxes outline the starting wound area. Graph (right) shows migration index (%) determined from the wound area measured with ImageJ, 48h following insert removal compared to 0h. Lower migration index indicates slower cell migration. **, P<0.001; *, P<0.017; **(E)** Extracellular (***, P<0.0001) and intracellular (**, P<0.001) lactate levels in LV_Control and LV_342 cells, shows increased extracellular lactate and decreased intracellular lactate in LV_342. **(F)** Intracellular lactate levels in LV_342 cells relative to LV_Control cells in three groups: untreated, 100uM AR-C141990 hydrochloride treated, or 100uM UK5099 treated. **, P<0.001, *, P<0.04. **(G)** Glucose uptake assay shows significantly lower uptake in LV_342 cells. **, P<0.001. Unless specified, data are mean±SEM; two-tailed Student's t-test.

Fig. 8. MiR-342-3p attenuates tumor growth, *in vivo*, in two additional mouse models of HCC. (A) Schematic of AAV-miR-342-3p treatment to study its effect on tumor development

in LT2/RAS mice (n=4 mice/group) and Xenograft tumor mice (LV_Control: n=4, LV_342: n=5). **(B)** Representative gross images of tumor-bearing LT2/RAS mice livers highlighting the tumor attenuating effect of AAV-342, two weeks post AAV treatment. AAV-342 relative to AAV-Control mice show **(C)** significantly decreased total liver/body weight (upper). *, P<0.04, and, validated miR-342-3p overexpression (lower) in their tumors. **, P<0.001. **(D)** Representative images (20x magnification) following Ki67 staining (upper) and TUNEL assay (lower). Corresponding graphs show % Ki67 positive cells (30 fields/treatment group) ***, P<0.0001, and average numbers of TUNEL positive cells/high power field (40 fields/treatment group). ***, P<0.0001. AAV-342 mice compared to AAV-Control mice show **(E)** significant decrease in MCT1 protein expression by western blot *, P<0.04, and, **(F)** significant increase in overall survival, as determined by Kaplan-Meier survival analysis (n=5 mice/group). Log-rank (Mantel-Cox) Test compared survival curves between treatment groups. P<0.0018. **(G)** Representative images (left) and average tumor volumes (right) of xenograft tumors from nude mice injected with AAV-342 compared to AAV-Control, measured every alternate day and sacrificed one week following AAV treatment. *, P<0.02 **, P<0.004, ***, P<0.0001. **(H)** AAV-342 xenograft tumors show significantly lower tumor weights (left) *, P<0.01 and, overexpression of miR-342-3p (right). ***, P<0.001. **(I)** Representative images (20x magnification) of Ki67 staining (upper) and TUNEL assay (lower) from AAV-Control and AAV-342 injected xenograft tumors. Corresponding graphs show % Ki67 positive cells (30 fields/treatment group) ***, P<0.0003 and average number of TUNEL positive cells/high power field (30 fields/treatment group). *, P<0.01 **(J)** Western blot shows significantly decreased relative MCT1 protein expression in AAV-342 xenograft tumors compared to AAV-Controls. ***, P<0.0001. Unless specified, data are mean±SEM; two-tailed Student's t-test.

Figure_1

Figure 1

Figure 2

Figure 3

Figure 4

Figure 4

AAV8-miR-342-3p or AAV8-Control
 5×10^{10} v.p, single i.v. injection

Figure_5

Figure 5

Figure 7

Figure 7

Figure_8

Figure 8

Highlights

MicroRNA-342-3p is a regulator of hepatocellular carcinoma regression.

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Highlights

- Distinct global microRNA landscape in regressing liver tumors compared to tumors.
- Identification and validation of miR-342-3p as a tumor suppressor in HCCs.
- MiR-342-3p significantly attenuates tumor development in three mouse models of HCC.
- MiR-342-3p prolongs survival of LT2/MYC and LT2/RAS mice with liver tumors.
- Identification of *Mct1*, as a target of miR-342-3p in HCC.
- We demonstrate a role for miR-342-3p in metabolic reprogramming by targeting *Mct1*.