Development of a 5-FU Modified miR-129 Mimic as a Therapeutic for Non-Small Cell Lung Cancer

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Abstract

Lung cancer is the leading cause of cancer-related deaths in the United States with non-small cell lung cancer (NSCLC) accounting for most cases. Despite advances in cancer therapeutics, the 5-year survival rate has remained poor due to several contributing factors, including its resistance to therapeutics. Therefore, there is a pressing need to develop therapeutics that can overcome resistance. Non-coding RNAs, including microRNAs (miRNAs), have been found to contribute to cancer resistance and therapeutics by modulating the expression of several targets involving multiple key mechanisms. In this study, we investigated the therapeutic potential of miR-129 modified with 5-fluorouracil (5-FU) in NSCLC. Our results show that 5-FU modified miR-129 (5-FU-miR-129) inhibits proliferation, induces apoptosis, and retains function as a miRNA in NSCLC cell lines A549 and Calu-1. Notably, we observed that 5-FU-miR-129 was able to overcome resistance to tyrosine kinase inhibitors and chemotherapy in cell lines resistant to erlotinib or 5-FU. Furthermore, we observed that the inhibitory effect of 5-FU-miR-129 can also be achieved in NSCLC cells under vehicle-free conditions. Finally, 5-FU-miR-129 inhibited NSCLC tumor growth and extended survival *in vivo* without toxic side effects. Altogether, our results demonstrate the potential of 5-FU-miR-129 as a highly potent cancer therapeutic in NSCLC.

Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States. Lung cancer is generally classified into two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer, with NSCLC accounting for ~85% of diagnosed cases.[1,2] Several treatment strategies have been developed to treat patients with NSCLC, including chemotherapy, radiotherapy, and targeted therapy.[2] For example, erlotinib is a tyrosine kinase inhibitor (TKI) used to treat patients diagnosed with NSCLC.[3-5] TKIs, including erlotinib, gefitinib, and osimertinib, specifically inhibit activating epidermal growth factor receptors (EGFRs) mutations.[4-8] Despite advancements in therapeutics to treat patients diagnosed with NSCLC, the 5-year survival rate after metastasis remains low (26%).[2,9] One of the contributing factors for this is NSCLC's resistance to cancer therapeutics.[3,10] For example, certain mutations of EGFR, including T790M, have been found to contribute to resistance to first generation TKIs erlotinib and gefitinib.[11-13] In addition to targeted therapeutics, resistance to chemotherapy in patients with NSCLC has also been well-documented.[3,10,14-17] Therefore, there is a need to develop new therapeutics that can overcome resistance to cancer therapies in NSCLC.

The dysregulation of microRNA (miRNA) expression has been found to contribute to the pathogenesis of cancer and its resistance to therapeutics.[18-21] MiRNAs are a class of small RNAs that are 21-25 nucleotides long and double-stranded. MiRNAs do not require complete complementary binding of their sequence with their mRNA targets, and instead, mature miRNAs bind to the 3' untranslated region (UTR) of their targets via complementary binding with their seed sequence. Therefore, miRNAs can target and affect the expression of multiple different mRNA sequences.[22-24] Because of their ability to downregulate multiple different targets, it has been reported that dysregulated expression of miRNAs can contribute to carcinogenesis, drug resistance, and stemness in several different cancers, including NSCLC.[23,25-28] Specifically, expression of hsa-miR-129-5p (miR-129) has been reported to be downregulated in patients with NSCLC.[29-32] Similarly, restoring miR-129 expression has been found to increase sensitivity to

chemotherapy in NSCLC cells.[27] These effects are, in part, due to the targets of miR-129, which

include apoptosis regulator Bcl-2 (Bcl-2) and high mobility group protein B1 (HMGB1).[30,33-35]

In this study, we demonstrate the therapeutic potential of miR-129 modified with the

chemotherapy drug 5-fluorouracil (5-FU) in NSCLC. By substituting uracil residues (U) within the

guide strand sequence of miR-129, we have a developed a 5-FU modified miRNA mimic, 5-FU-

miR-129 with the ability to be delivered to cells without the aid of a transfection vehicle. We

observed that 5-FU-miR-129 inhibited cell proliferation, induced apoptosis, and was able to be

delivered without the use of a transfection vehicle in NSCLC cell lines A549 and Calu-1. In addition,

5-FU-miR-129 retained miR-129's target specificity and miRNA function. We also observed 5-FU-

miR-129's ability to overcome resistance to the EGFR TKI, erlotinib, as well as to 5-FU itself. We

also identified that 5-FU-miR-129 has a pleiotropic effect on cell death genes, including *FAS* and

DPYSL4, and death signaling pathways such as the NF-κB signaling pathway. Finally, we

examined 5-FU-miR-129's therapeutic potential compared to nucleoside analog chemotherapy

drugs 5-FU and gemcitabine (Gem) *in vivo* and observed a significant inhibition of NSCLC tumor

growth and an increase in median survival. As a result, we demonstrate that 5-FU-miR-129 has

potential as a therapeutic agent that can overcome resistance to cancer therapeutics in NSCLC.

Results

5-FU-miR-129 Inhibits Cell Proliferation, Induces Apoptosis and Cell Cycle Arrest in NSCLC

We investigated the efficacy of 5-FU-miR-129 on NSCLC cell lines A549 and Calu-1. As

shown previously, we have developed 5-FU-miR-129 by substituting the uracil residues of miR-

129's guide strand with 5-FU (Figure 1A).[34] We assessed the effects of 5-FU-miR-129 on cell

proliferation in A549 and Calu-1 cells (Figure 1B & Figure S1). At 50 nM, 5-FU-miR-129 inhibited

proliferation by 82.69% in A549 cells and by 68.31% in Calu-1 cells six days post-transfection

(Figure 1B). In addition, the unmodified miR-129 was found to only inhibit proliferation in A549

cells (53.20%), but not in Calu-1 cells.

We also assessed the effects of 5-FU-miR-129 on apoptosis and the cell cycle. 5-FU-miR-

129 induced apoptosis in both NSCLC cell lines A549 and Calu-1 with a 7.2-fold and 2.9-fold

increase of apoptotic cells, respectively (Figure 1C). In addition, unmodified miR-129 induced

apoptosis with a 2.4 and 1.7-fold increase of apoptotic cells in A549 and Calu-1, respectively.

Surprisingly, siBCL2 only induced an increase in apoptotic cells in A549 cells (1.5-fold increase).

Furthermore, 5-FU-miR-129 induced an increase in the G1/S phase ratio in A549 cells,

suggesting that 5-FU-miR-129 induces G1 cell cycle arrest in A549 cells (Figure 1D). In contrast,

5-FU-miR-129 did not induce a significant increase in the G1/S phase ratio in Calu-1 cells.

5-FU-miR-129 Downregulates Expression of Bcl-2 and HMGB1 in NSCLC

To demonstrate that 5-FU-miR-129 retains its function as a miRNA mimic of miR-129, we

investigated 5-FU-miR-129's ability to downregulate miR-129 targets Bcl-2 and HMGB1. *BCL2*

and *HMGB1* have both been reported to be targets of miR-129.[30,33] Therefore, we reasoned that

5-FU-miR-129 would also target *BCL2* and *HMGB1* (Figure 2A & 2C). In addition, 5-FU-miR-129

has previously been reported to downregulate Bcl-2 in colorectal cancer (CRC).[34] 5-FU-miR-129

only downregulated Bcl-2 expression in A549 cells (Figure 2B). Unmodified miR-129 also

downregulated Bcl-2 expression in both A549 and Calu-1 cells while 5-FU alone did not induce a

significant change in Bcl-2 expression. The combination of unmodified miR-129 and 5-FU only

downregulated Bcl-2 expression in A549 cells. In contrast, 5-FU-miR-129 downregulated HMGB1

expression in both A549 and Calu-1 cells (Figure 2D). Furthermore, HMGB1 expression was

downregulated by miR-129, and the combination of unmodified miR-129 and 5-FU treatment

conditions in both A549 and Calu-1 cells. Like its effect on Bcl-2, 5-FU was not found to have a

significant effect on HMGB1 expression.

Due to the observed effects of 5-FU-miR-129 on cell cycle control only in A549 cells, we

investigated the effects of 5-FU-miR-129 on the expression of cellular tumor antigen p53 (p53)

and cyclin-dependent kinase inhibitor 1 (p21). 5-FU-miR-129 upregulated the expression of both

p53 and p21 in A549 (Figure S2A). Treatment with siHMGB1 downregulated p53 expression while

unmodified miR-129, or a combination of unmodified miR-129 and 5-FU, upregulated p21

expression. 5-FU alone did not have a significant effect on the expression of either p53 or p21 in

both A549 and Calu-1 cells. Consistent with previous reports of a p53 deletion, [36] we did not

observe expression of p53 (Figure S2B). Expression of p21 was also not observed in Calu-1 cells.

5-FU-miR-129 Promotes Autophagy in NSCLC in a Cell-Line Dependent Manner

Autophagy has been reported to have an intricate role in cell death, and autophagy has

been reported be regulated by p21 and p53.[37-39] Therefore, we investigated the effects of 5-FU-

miR-129 on autophagy in NSCLC. A control with fresh serum (Negative Control (+Serum)) and a

control under serum-starved conditions (Negative Control (Serum-Starved)) were used to

represent inhibition and induction of autophagy, respectively (Figure 3A & 3B). Using the marker

LC3B and the expression ratio between LC3B-II and LC3B-I to measure autophagy, 5-FU-miR-

129 did not induce a significant change to autophagy in A549 cells (Figure 3A). In contrast, 5-FU-

miR-129 promoted autophagy in Calu-1 cells (Figure 3B).

Vehicle-Free Delivery of 5-FU-miR-129 in NSCLC In Vitro

In this study, we investigated 5-FU-miR-129's ability to be delivered without the aid of a

transfection vehicle in NSCLC cells. Under vehicle-free conditions, 5-FU-miR-129 downregulated

both Bcl-2 and HMGB1 in A549 cells (Figure 4A). The respective siRNAs (siBCL2 and siHMGB1),

unmodified miR-129, and 5-FU did not induce a significant change in Bcl-2 or HMGB1 expression.

Furthermore, we observed that vehicle-free transfection of 5-FU-miR-129 can inhibit cell

proliferation. Six days post-transfection, 5-FU-miR-129 inhibited proliferation by 84.69% in A549

cells (Figure 4B).

5-FU-miR-129 Overcomes Erlotinib and Chemoresistance in NSCLC

To demonstrate 5-FU-miR-129's impact on resistance in NSCLC, we investigated the

ability of 5-FU-miR-129 to inhibit proliferation in cells resistant to the EGFR TKI erlotinib. Using a

cell line that is resistant to erlotinib, HCC827ER, we measured the IC_{50} of erlotinib in HCC827ER

cells (64.26 nM), which is approximately 2.9 to 9.9-fold higher than previously reported values in

its parental line (HCC827, 6.5 – 22 nM)[40,41] and demonstrates HCC827ER's resistance to erlotinib

(Figure 5A). Compared to the IC_{50} of erlotinib (64.26 nM), we observed a 3.7-fold decrease in the

IC_{50} of 5-FU-miR-129 (17.59 nM) in HCC827ER cells (Figure 5A). We then monitored 5-FU-miR-

129's ability to inhibit proliferation (Figure 5B). Six days post-transfection, 5-FU-miR-129 inhibited

proliferation by 90.03% in HCC827ER cells at 50 nM. In addition, unmodified miR-129 inhibited

proliferation by 27.67% in HCC827ER cells at 50 nM.

We also examined 5-FU-miR-129's potency and ability to inhibit proliferation in cells

resistant to 5-FU (A549-5FU). To confirm that A549-5FU acquired resistance to 5-FU, we

measured the IC_{50} of 5-FU in both A549-5FU and A549 cells. The IC_{50} of 5-FU increased by 2.5-

fold in A549-5FU cells (29.57 μM) compared to A549 cells (11.67 μM) (Figure 5C). We then

measured the IC_{50} of 5-FU-miR-129 in A549-5FU cells and observed that the IC_{50} of 5-FU-miR-

129 was 837-fold lower than the IC_{50} of 5-FU (29.57 μM). We then examined 5-FU-miR-129's

ability to inhibit proliferation in A549-5FU cells (Figure 5D). Six days post-transfection, 5-FU-miR-

129 inhibited proliferation by 75.56% in A549-5FU cells at 50 nM. In addition, unmodified miR-

129 inhibited proliferation by 73.37% and the combination of miR-129 and 5-FU inhibited

proliferation by 90.77%. In contrast, at the IC_{50} of 5-FU in A549 cells (11.67 μM), 5-FU alone in

did not induce a significant change in proliferation in A549-5FU cells.

All IC_{50} values were less than 50 nM in several NSCLC cells lines, demonstrating the

efficacy of 5-FU-miR-129 at a low nM range (Table 1). We also compiled a table of IC_{50} values

for the chemotherapy nucleoside analog agents 5-FU and Gem, and platinum-based agent

cisplatin in the NSCLC cell line A549 and compared them to 5-FU-miR-129's IC_{50} value (Table

S1). The IC_{50} of 5-FU-miR-129 (13.33 nM) was 37.4-fold and 853-fold less than the IC_{50} of Gem

(458.48 nM)[42] and cisplatin (11.37 μM), respectively. Our results show that 5-FU-miR-129 is at

least 30-fold more effective than chemotherapy agents.

Identifying Changes in mRNA Expression of Cell Death Pathway Induced by 5-FU-miR-129

We investigated changes in the global impact of cell death genes and pathways via RNA

expression analysis after treatment with 5-FU-miR-129 in A549 (Table S2) and Calu-1 cells (Table

S3). Of note, *ULK1* was found to be consistently downregulated in both A549 and Calu-1.

Consistent with our western blot data, we also observed downregulated expression of *BCL2* only

in A549, but not in Calu-1 cells. In contrast, *BCL2A1, CYLD, DPYSL4, FAS, and GADD45A* were

found to be consistently upregulated in both A549 and Calu-1 cells. Using GeneSpring GX

(Agilent), direct interactions between upregulated and downregulated targets were analyzed

(Figure 6A & 6C). Pathway analysis of identified targets was also performed via Enrichr using the

KEGG 2021 Human Database (Figure 6B & 6D).[43-45] Of note, changes in both the apoptosis and

NF-κB signaling pathway were found to be associated with 5-FU-miR-129 in A549 and Calu-1

cells.

Therapeutic Potential of 5-FU-miR-129 in NSCLC In Vivo

To demonstrate the therapeutic potential of 5-FU-miR-129 in NSCLC *in vivo*, we examined

the effects of 5-FU-miR-129 on a luciferase-expressing NSCLC cell line (A549-Luc) in NOD-SCID

mice. Compared to the vehicle control (negative) group, decreased luciferase expression was

observed in mice treated with 5-FU-miR-129 (Figure 7A). By day 37, a significant decrease in

tumor growth was observed in mice treated with 5-FU-miR-129 (Figure 7B & 7C). Compared to

the increase in tumor growth in the negative control group (13.22-fold growth), 5-FU-miR-129

induced a greater than 4.9-fold reduction in tumor growth in mice (2.67-fold growth, $p = 0.0008$).

In addition, Gem decreased tumor growth (5.74-fold, p = 0.0049), but 5-FU did not induce a

significant change in tumor growth in mice. We also monitored and compared survival between

the different treatment groups (Figure 7D). Compared to the negative control group, only 5-FU-

miR-129 induced an increase in the median survival for mice from 86 days to 100 days (p = 0.0391,

hazard ratio = 0.3209). Hair loss and significant loss in body weight were also not observed in

mice treated with 5-FU-miR-129 (data not shown).

Discussion

In this study, we developed 5-FU-miR-129 as a potential effective cancer therapeutic in

NSCLC. This is highly consistent with our recent findings that 5-FU-modified miRNAs can serve

as a platform miRNA-based therapeutic technology in several different cancer types, including

the therapeutic potential of 5-FU-miR-129 in CRC.[34,46-49] As seen by its ability to inhibit

proliferation and induce apoptosis in NSCLC cell lines A549 and Calu-1 (Figure 1), our results

demonstrate 5-FU-miR-129's potential in NSCLC.

Our results also suggest that 5-FU-miR-129 retains its function as a miRNA, specifically,

its ability to pleiotropically knock down expression of miR-129 targets, HMGB1 and Bcl-2. Our

previous studies have demonstrated that miR-129 and 5-FU-miR-129 can downregulate Bcl-2 in

CRC [33,34]. Furthermore, HMGB1 and Bcl-2 both have been reported to be promising targets in

cancer therapeutics, and both have been reported to promote autophagy and resistance to

chemotherapy in NSCLC.[30,50-55] Bcl-2 is a well-studied protein that has been reported to inhibit

apoptosis,[56] and several Bcl-2 inhibitors have been developed.[57] We also observed that 5-FU-

miR-129 did not downregulate Bcl-2 expression in Calu-1 cells (Figure 2B), which may be due to

a difference in the abundance of Bcl-2 mRNA transcript levels. Although Bcl-2 was not knocked

down in Calu-1 cells, we observed that 5-FU-miR-129 induced a significant increase in apoptotic

cells in both A549 and Calu-1. This is not surprising as miRNA targets are dependent on the

cellular context.[58] Furthermore, despite observing a knock down of Bcl-2 via siBCL2 (Figure 2B),

siBCl2 did not induce an increase in apoptotic cells (Figure 1C). This suggests that 5-FU-miR-

129 may induce apoptosis through a pathway that may not be dependent on Bcl-2. We also

reasoned that knock down of HMGB1 by 5-FU-miR-129 would impact autophagy. Surprisingly,

we observed that knock down of HMGB1 by 5-FU-miR-129 did not affect autophagy in A549 cells

and increased autophagy in Calu-1 cells. 5-FU-miR-129's dependence on cellular context for

target selection may also influence 5-FU-miR-129's effect on autophagy.

232  To better understand the impact of 5-FU-miR-129 in NSCLC cells, we quantified changes

in mRNA expression of genes and pathways related to cell death mechanisms. These results are

highly consistent with the pleiotropic effects of 5-FU-miR-129 in NSCLC in triggering apoptosis

and autophagy. *FAS* and *GADD45A* have been reported to play a role in inducing apoptosis and

have also been previously identified to have a positive correlation with patient prognosis in

patients with NSCLC.[59-63] *DPYSL4* has been reported to function as a tumor suppressor,[64] and

interestingly, *DPYSL4* has also been reported to play a role in inducing necrosis.[65] However,

necrosis was not an area of focus for this study, and 5-FU-miR-129's role in necrosis can be

examined in future studies. Consistent with our western blot results, *BCL2* was observed to only

be downregulated in A549, but not in Calu-1 cells. Furthermore, pathway analysis of the observed

changes in mRNA expression suggests that 5-FU-miR-129 primarily affects the apoptosis

pathway in both A549 and Calu-1 cells (Figure 6B & 6D). In addition, the NF-κB signaling pathway

was suggested to be affected by 5-FU-miR-129, which has been reported to play a major role in

inflammation and cancer, and HMGB1 has also been reported to activate this pathway.[66-68]

Therefore, examining 5-FU-miR-129's role in the NF-κB signaling pathway may be promising. 5-

FU-miR-129's effect on global changes in mRNA expression and protein abundance can also be

further elucidated with RNA sequencing and proteomic studies.

249  Currently, the development and refinement of oligonucleotide delivery has been an area

of study in RNA-based therapeutics.[46,69] The inherent toxicity and unforeseen side effects of some

delivery vehicles have been an obstacle in the effective delivery of RNA-based therapeutics.[46,70,71]

Our results demonstrate that 5-FU-miR-129 can be delivered to NSCLC cell without the aid of a

delivery vehicle via knock down of Bcl-2 and HMGB1 expression and inhibition of proliferation

(Figure 4). This is consistent with our previous studies that have shown that 5-FU-modified

miRNAs can be delivered without the use of a transfection vehicle in pancreatic, gastric, lung, and

breast cancer.[46-49] In addition, this is consistent with previous studies that show that 5-FU-miR-

129, specifically, inhibits proliferation in CRC via vehicle-free delivery.[34] Notably, 5-FU is not listed

among the list of current treatments for patients with NSCLC.[72] Although 5-FU is not currently

used, our results demonstrate that the 5-FU modification is necessary for miR-129 to enter cells

without the aid of a delivery vehicle. Because the inhibitory effect of 5-FU-miR-129 can be

achieved under vehicle-free conditions, we believe that this feature can overcome a key obstacle

of RNA-based therapeutics.

This study also revealed cell line-dependent differences that were not observed in

previous studies. Unlike previous studies, 5-FU-miR-129 was shown to have different effects on

cell cycle arrest, autophagy, and knock down in miR-129 targets between A549 and Calu-1 cells.

We hypothesize that several factors could have contributed to the differences observed in our

study. A549 expresses wild-type p53 and Calu-1 is a p53-null mutant. This can lead to a difference

of expression of downstream targets of p53, as demonstrated by a difference in expression of

p21 between A549 and Calu-1 cells. Therefore, this difference can also affect pathways related

to cell death and survival, including cell cycle arrest and autophagy.[34,38,73,74] Interestingly, this

could also contribute to the difference in induced apoptotic cells observed between A549 and

Calu-1 cells due to the induction of autophagy in Calu-1 cells. Furthermore, although A549 and

Calu-1 are both classified as NSCLC, A549 is also classified under the adenocarcinoma subtype

while Calu-1 is classified under the squamous cell carcinoma subtype. Despite both subtypes

being classified as NSCLC, the difference in subtype can contribute to differences in clinical

decisions, including treatment.[75,76] Therefore, 5-FU-miR-129's relationship with p53 and NSCLC

subtypes should be investigated in future studies. However, despite the different genetic

backgrounds in NSCLC cells, we demonstrate that 5-FU-miR-129 inhibits proliferation, induces

apoptosis, retains miRNA function, and can be delivered vehicle-free in NSCLC *in vitro*.

Importantly, as a potential therapeutic for NSCLC that can overcome resistance to cancer

therapeutics, we demonstrate that 5-FU-miR-129 can overcome resistance to chemotherapy and

TKIs. EGFR TKIs are currently being used in a clinical setting for patients diagnosed with NSCLC,

but patients commonly develop resistance to TKIs.[3-5,11,75,76] Our results demonstrate that 5-FU-

miR-129 can provide a foundation in using 5-FU-miR-129 to overcome erlotinib resistance in the

development of future therapeutics for NSCLC. In addition to TKIs, *KRAS* inhibitors targeting the

G12C mutation were approved for treating NSCLC.[77,78] A549 and Calu-1 are NSCLC cell lines

with *KRAS* mutations, specifically G12S (A549) and G12C (Calu-1) with G12C being the most

common *KRAS* mutant variant in NSCLC.[79] However, resistance to these inhibitors have also

been observed.[80,81] *KRAS* is one of the predicted targets of miR-129 found through the target

prediction software, TargetScan (data not shown), and we reason that 5-FU-miR-129 may also

have the potential to overcome resistance to *KRAS* inhibitors.[82] In addition to targeted inhibitors,

platinum-based chemotherapies, including cisplatin, are used in a clinical setting for patients

diagnosed with NSCLC.[83] Compared to chemotherapy agents 5-FU, Gem, and cisplatin, 5-FU-

miR-129 demonstrates potential as an efficacious therapeutic by exhibiting nanomolar potency

and overcoming resistance to chemotherapy.

We also demonstrate 5-FU-miR-129's promising potential as a cancer therapeutic in

NSCLC within an *in vivo* context. Compared to the negative control group and 5-FU alone group,

5-FU-miR-129 was able to inhibit NSCLC tumor growth and increase survival (Figure 7). In

addition, we did not observe any noticeable declines in animal health (e.g., hair loss and body

weight loss; data not shown), thus suggesting 5-FU-miR-129's potential to avoid side effects

associated with cancer therapeutics. Interestingly, the mice in this study were treated with 5-FU-

miR-129 at 3.75 mg/kg per injection. The 5-FU-miR-129 treated group was given a dose that is

over 13-fold less than the 5-FU (50 mg/kg) and Gem (50 mg/kg) treated groups. This suggests

that 5-FU-miR-129 is also effective at a lower dose than other chemotherapy drugs *in vivo*.

In summary, our studies demonstrate 5-FU-miR-129's therapeutic potential in NSCLC by

inhibiting cell proliferation, induces apoptosis, and can overcome resistance to both

chemotherapy and targeted therapy. Our studies demonstrate that 5-FU-miR-129 exhibits high

potency compared to therapies that are currently in clinical use for NSCLC. We demonstrate that

5-FU-miR-129 impacts promising targets Bcl-2 and HMGB1, along with other critical genes and

pathways related to cell death. 5-FU-miR-129 is also able to be delivered to NSCLC cell without

the aid of a delivery vehicle, a unique feature for nucleic acid-based medicine to reduce potential

toxicity associated with the delivery vehicle. Finally, we also demonstrate the proof-of-concept

that 5-FU-miR-129 can inhibit NSCLC tumor growth *in* vivo while increasing survival without toxic

side effects. This study establishes the foundation to further develop 5-FU-miR-129 as an

effective therapeutic for treating NSCLC.

Materials and Methods

Cell Lines and Culture

Human non-small cell lung cancer cell lines (A549 and Calu-1) were purchased from the

American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Ham's F-12K

(Kaighn's) Medium or McCoy's 5A (Modified) Medium (Thermo Fisher Scientific, Waltham, MA,

USA) supplemented with 10% fetal bovine serum (FBS) (MilliporeSigma, St. Louis, MO, USA).

FBS was sterilized via Steriflip-GP, 0.22 μm (MilliporeSigma, St. Louis, MO, USA) prior to addition

to media.

The resistant cell line A549 (5-FU) was generated by treating the A549 cell line with 5-

fluorouracil (MillporeSigma, St. Louis, MO, USA) starting at the $IC_{12.5}$ for 48 hours in Ham's F-12K

(Kaighn's) Medium supplemented with 10% dialyzed fetal bovine serum (DFBS) (Thermo Fisher

Scientific, Waltham, MA, USA). After 48 hours, media was changed to fresh media until plates

recovered from treatment. This process was repeated with increasing concentrations 5-

fluorouracil. DFBS was sterilized via Steriflip-GP, 0.22 μm (MilliporeSigma, St. Louis, MO, USA)

prior to addition to media. The erlotinib-resistant cell line HCC827ER was given as a gift from Dr.

John D. Haley (Renaissance School of Medicine at Stony Brook University). HCC827ER was

maintained in RPMI-1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented

with 10% FBS and 1 μM erlotinib (MedChem Express, South Brunswick Township, NJ, USA).

The luciferase-expressing lung cancer line, A549-Luc, was generated by transfecting the

A549 line with a luciferase-expressing lentiviral construct (gift from the Y. Ma laboratory at

Renaissance School of Medicine at Stony Brook University). After transfection with the lentiviral

construct, the line was maintained in Ham's F-12K (Kaighn's) Medium supplemented with 10%

FBS and 1 ug/ml puromycin (MilliporeSigma, St. Louis, MO, USA). Luciferase expression was

confirmed via IVIS before the cell line was used for *in vivo* experiments.

Transfection

Cell lines were transfected with Oligofectamine™ (Thermo Fisher Scientific, Waltham, MA,

USA) and oligonucelotides as previously described.[34,46] In brief, A549, Calu-1, A549 (5-FU), and

HCC827ER cell lines were seeded onto 6-well plates at a cell density of 1 x 10^5 cells/well. 24

hours later, cells were transfected with Oligofectamine™ and their respective oligonucleotides

according to the manufacturer's protocol. 24 hours post-transfection, 5-FU was added to their

respective wells. All oligonucleotides were transfected at a concentration of 50 nM. 5-FU was

either added at 450 nM, a concentration equivalent to 5-FU in 5-FU-miR-129, or at 11.67 μM, the

IC_{50} of 5-FU in A549.

Vehicle-free transfections were performed as previously described. In brief, cell lines were

seeded onto 6-well plates at 1 x 10^5 cells/well. 24 hours after seeding, the cells were transfected

with their respective oligonucleotides by directly adding the oligonucleotides to their respective

wells. 24 hours post-transfection, media was changed to fresh media supplemented with 10%

DFBS and 5-FU was added to their respective wells.

5-FU-miR-129 was purchased from Horizon Discovery (Horizon Discovery, Cambridge,

UK) as previously described. In brief, hsa-miR-129-5p's sequence was modified by substituting

uracil residues with 5-FU. Hsa-miR-129-3p's sequence was left unmodified to avoid potential off-

target effects and to preserve miRNA function. The two strands were then annealed according to

the manufacturer's protocol. A non-specific scramble miRNA, Pre-miR™ Negative Control #2

(Thermo Fisher Scientific, Waltham, MA, USA) was used to represent the negative control.

siBCL2 (Smart Pool) and siHMGB1 (Smart Pool) were purchased from Horizon Discovery

(Horizon Discovery, Cambridge, UK). Pre-miR-129-5p was purchased from Thermo Fisher

Scientific (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Proliferation Analysis

For treatment studies with vehicle (Oligofectamine™), cell lines were seeded onto 6-well

plates at 1 x 10^5 cells/well and transfected with their respective oligonucleotides as described

above. 24 hours post-transfection, cells were trypsinized and re-seeded onto 96-well plates at

1000 cells/well in triplicate per condition in fresh media supplemented with 10% DFBS. 5-FU was

also added to their respective wells. Cell viability was measured using WST-1 Cell Proliferation

Reagent (MilliporeSigma, St. Louis, MO, USA) according to the manufacturer's protocol. In brief,

cells were incubated in 100 μl fresh media supplemented with 10% DFBS and 10 μl WST-1 for 1

hour at 37°C. After incubation, absorbance was measured at 450 nm and 630 nm with O.D.

calculated by subtracting absorbance at 630 nm from absorbance at 450 nm. Absorbances were

then normalized to the negative control. Cell viability was measured at their respective time points

(1, 3, and 6 days post-transfection).

378   For treatment studies without a transfection vehicle, cell lines were seeded onto 96-well

379 plates at 1000 cells/well in triplicate per condition. 24 hours after seeding cells onto plates, media

380 was changed to oligonucleotides mixed with fresh media supplemented with 10% DFBS for their

381 respective conditions. 24 hours post-transfection, media was changed to fresh media

382 supplemented with 10% DFBS and 5-FU was added to their respective wells. Cell viability was

383 measured using WST-1 Cell Proliferation Reagent as described above.

385 *Cytotoxicity Assay*

386   Cytotoxicity of 5-FU-miR-129 was measured by transfecting cell lines as described above

387 with varying concentrations of 5-FU-miR-129 in 6-well plates. Cells were then re-seeded onto 96-

388 well plates. 6 days post-transfection, cell viability was measured with WST-1 Cell Proliferation

389 Reagent as described above. IC_{50} values were calculated using GraphPad Prism 9 (GraphPad

390 Software, San Diego, CA, USA).

391   Cytotoxicities of chemotherapy drugs 5-FU, cisplatin (MilliporeSigma, St. Louis, MO, USA)

392 and erlotnib were measured by seeding cells onto 96-well plates at 1000 cells/well per drug

393 concentration. 24 hours post-seeding, media was changed to fresh media supplemented with 10%

394 DFBS and their respective concentrations of drugs. 48 hours post-treatment, media was changed

395 with fresh media supplemented with 10% DFBS. 6 days post-treatment, cell viability was

396 measured with WST-1 Cell Proliferation Reagent as described above. IC_{50} values were calculated

397 using GraphPad Prism 9.

399 *Western Immunoblot Analysis*

400   Cell lines were seeded and transfected with or without a transfection vehicle

401 (Oligofectamine™) in 6-well plates with their respective conditions as described above. 3 days

402 post-transfection, cells were lysed with a mixture of RIPA buffer (MilliporeSigma, St. Louis, MO,

403 USA) and protease inhibitor cocktail (MilliporeSigma, St. Louis, MO, USA) and protein samples

were collected for western immunoblot analysis. Proteins were probed with mouse anti-Bcl-2

antibody (Thermo Fisher Scientific, MA511757, 1:100), rabbit anti-HMGB1 antibody (Cell

Signaling, 3935S, 1:500), mouse anti-p21 antibody (Cell Signaling, 2946S, 1:500), rabbit anti-p53

antibody (Thermo Fisher Scientific, BS-8687R, 1:500), rabbit anti-LC3B (Cell Signaling, 3868S,

1:1000), or mouse anti-β-actin antibody (MilliporeSigma, A5441, 1:10,000,000). Bcl-2, p21, p53

and β-actin primary antibodies were diluted in 5% milk (Bio-Rad, Hercules, CA, USA) in TBST.

HMGB1, and LC3B primary antibodies were diluted in 5% BSA (MilliporeSigma, St. Louis, MO,

USA) in TBST. After staining with primary antibodies, proteins were probed with secondary

antibodies goat anti-mouse-HRP (Bio-Rad, 1706516, 1:5000) or goat anti-rabbit-HRP (Bio-Rad,

1721019, 1:5000) with their respective primary antibodies. Proteins were then treated with

SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham,

MA, USA) and visualized with a LI-COR Biosciences Odyssey Fc imaging system. Protein bands

were quantified with Image Studio, Version 5.2.5 (LI-COR Biosciences, Lincoln, NE, USA).

Autophagy Analysis

3 days post-transfection, cells were washed with PBS and media was changed to either

fresh media or Earle's Balanced Salt Solution (EBSS) (Thermo Fisher Scientific, Waltham, MA,

USA) in order to either inhibit (Negative Control (+Serum)) or induce autophagy (Negative Control

(Serum-Starved), respectively. Cells were then incubated for 4 hours at 37°C before protein

samples were collected for western immunoblot analysis as described above. Autophagy was

measured via the expression ratio of LC3B-II/LC3B-I.

Cell Cycle Analysis

3 days post-transfection, cells were resuspended at 0.5 to 1 x 10^6 cells/ml in Krishan

modified buffer supplemented with 0.02 mg/ml RNase H (Thermo Fisher Scientific, Waltham, MA,

USA) and 0.05 mg/ml propidium iodide (MilliporeSigma, St. Louis, MO, USA). Cells were then

analyzed by flow cytometry via CytoFLEX Flow Cytometer (Beckman Coulter, Brea, CA, USA)

and results were analyzed by Modfit LT Software (BD Biosciences, Sparks, MD, USA).

Apoptosis Assay

3 days post-transfection, cells were resuspended in annexin-binding buffer (Thermo

Fisher Scientific, Waltham, MA, USA). Cells were then stained with Annexin V (Thermo Fisher

Scientific, Waltham, MA, USA) and propidium iodide (MilliporeSigma, St. Louis, MO, USA).

Stained cells were analyzed by flow cytometry via CytoFLEX Flow Cytometer (Beckman Coulter,

Brea, CA, USA).

Cell Death Profiler Assay

3 days post-transfection, total RNA was extracted from transfected cells using TRIzol

reagent (Thermo Fisher Scientific, Waltham, MA, USA). From the extracted total RNA, cDNA was

prepared using QIAGEN's RT^2 First Strand Kit (QIAGEN, Hilden, Germany). RT-PCR was

performed using QIAGEN's RT^2 Profiler™ PCR Array Human Cell Death PathwayFinder

(QIAGEN, Hilden, Germany). RT-PCR data was analyzed and visualized for pathway analysis by

GeneSpring GX 14.9 (Agilent Technologies, Santa Clara, CA, USA) and by Enrichr (Icahn School

of Medicine, Mount Sinai, NY, USA).[43-45]

Mouse Non-Small Cell Lung Cancer Metastasis Model

All animal procedures were approved by the Stony Brook University Institutional Animal

Care and Use Committee (IACUC). Non-obese diabetic (NOD)/severe combined

immunodeficiency (SCID) mice were purchased from The Jackson Laboratory (The Jackson

Laboratory, Bar Harbor, ME, USA). 8-week-old NOD/SCID mice (2 – 3 males and 2 – 3 females

per treatment group) were injected with 1 x 10^7 A549 (+Luc) cells via intravenous (IV) tail vein

injection. Mice were then divided into four groups (control, 5-FU, gemcitabine, and 5-FU-miR-

129). Control and 5-FU-miR-129 groups were treated with either 75 μg of PEI-MAX (control)

(Polysciences, Warrington, PA, USA) or 75 μg PEI-MAX + 75 μg 5-FU-miR-129 (5-FU-miR-129).

Average mouse weight ≈ 20 g, 75 μg injection/20 g weight = 3.75 mg/kg. 5-FU and gemcitabine

(Gem) groups were treated with either 50 mg/kg 5-FU (5-FU) or 50 mg/kg Gem (MilliporeSigma,

St. Louis, MO, USA). In the first treatment session (Treatment 1), control and 5-FU-miR-129

groups were treated via IV tail vein injection every other day for 2 weeks (eight times), and 5-FU

and gemcitabine groups were treated via intraperitoneal (IP) injection every 3 days for 2 weeks

(four times). In the second treatment session (Treatment 2), control and 5-FU-miR-129 groups

were treated via IP injection every other day for 1 week (four times), and 5-FU and Gem groups

were treated via IP injection every 3 days for 1 week (two times). Treatment sessions started at

2 days post-injection of A549 (+Luc) cells (Day 2, Treatment 1) and 33 days post-injection (Day

33, Treatment 2). 10 minutes prior to measuring luciferase expression via luciferase activity, mice

were injected with RediJect D-Luciferin (PerkinElmer, Waltham, MA, USA). Luciferase expression

was used to measure tumor growth using the IVIS Spectrum *In Vivo* Imaging System (IVIS)

(PerkinElmer, Waltham, MA, USA). Tumor growth was calculated as a function of (total flux at

time of measurement [p/s])/(total flux at initial measurement [p/s]). Mice were monitored daily and

euthanized according to IACUC guidelines to determine survival time.

Statistical Analysis

All experiments were performed at least three times, and all statistical analyses was

performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Statistical

significance between two groups was determined using Student's t-test. Data is presented as

mean $\pm$ SD. Statistical significance was presented either as described in the figure legends or

with the following asterisks (*p < 0.05; **p < 0.01; *** p < 0.001; **** p < 0.0001).

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Declaration of Interests

A.F. and J.J. have filed a patent for 5-FU modified miRNA mimetics. J.J. is a scientific co-founder of Curamir Therapeutics. The remaining authors declare no competing interests.

Author Contributions

Conceptualization, G.H., and J.J; Methodology, G.H. and J.J; Investigation, G.H., J.G.Y., A.F., H.F.; Writing – Original Draft, G.H. and J.J.; Writing – Reviewing & Editing, G.H., J.G.Y., A.F., J.D.H., and J.J.; Supervision, J.J.; Project Administration, J.J.; Funding Acquisition, J.J.

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779 **Tables**

780 **Table 1: IC$_{50}$ Values of 5-FU-miR-129 in NSCLC Cell Lines.**

Cell Line	5-FU-miR-129 (nM)
A549	13.33
Calu-1	13.03
A549 (5-FU)	35.32
HCC827ER	17.59

Figure Legends

Figure 1: 5-FU-Modified miR-129 Mimic Demonstrates Therapeutic Potential by Inhibiting

Proliferation, Inducing Apoptosis and Inducing Cell Cycle Arrest in NSCLC *In Vitro*. (A) A

5-FU-modified miR-129 mimic (5-FU-miR-129) was developed by substituting the uracil residues

within miR-129-5p's sequence with 5-fluorouracil (green, 5-FU). 5-FU is a nucleoside analog of

uracil with fluorine replacing the hydrogen on the fifth carbon of the uracil ring (*). (B) 5-FU-miR-

129 inhibits proliferation in NSCLC *in vitro* 1, 3, and 6 days post-transfection in A549 cells. 1, 3,

and 6 days post-transfection in Calu-1 cells is available in Figure S1. 6 days post-transfection, 5-

FU-miR-129 inhibited cell proliferation by 82.69% in NSCLC cell lines A549 ($p < 0.0001$) and by

68.31% in Calu-1 ($p = 0.0006$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p < 0.0001$ (n = 3). (C) 5-FU-

miR-129 induced apoptosis *in vitro* with a 7.2-fold increase in A549 cells ($p = 0.0020$) and a 2.9-

fold increase in Calu-1 cells ($p = 0.0240$). *$p < 0.05$, **$p < 0.01$ (n = 3) (D) 5-FU-miR-129 induced

cell cycle arrest in a cell-line dependent manner *in vitro*. Cell cycle arrest was only observed in

A549 cells, as seen by a loss of cells in S phase and an increase in G1/S phase ratio in cells

treated with 5-FU-miR-129 (28.05, $p = 0.0288$) compared to the negative control (2.211). A

significant change in cell cycle arrest was not observed in Calu-1. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

(n=3). All transfections were performed with 50 nM of negative control, siRNA controls, unmodified

miR-129, or 5-FU-miR-129. Cells were also treated with 450 nM of 5-FU to mimic the equivalent

concentration of 5-FU in 5-FU-miR-129. Data are represented as mean ± SD.

Figure 2: 5-FU-miR-129 Retains miRNA Function and Knocks Down Expression of Bcl-2

and HMGB1. (A) 5-FU-miR-129 can potentially bind to miR-129's predicted binding site within the

3' UTR of *BCL2*. (B) 5-FU-miR-129 retains miRNA function as seen by a knock down of Bcl-2

expression in A549 cells ($p = 0.0006$). A knock down of Bcl-2 expression was not observed in

Calu-1 cells. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (n = 3). (C) 5-FU-miR-129 can also potentially

bind to miR-129's four predicted binding sites within the 3' UTR of *HMGB1*. (D) 5-FU-miR-129

was observed to knock down HMGB1 expression in both A549 ($p < 0.0001$) and Calu-1 cells ($p < 0.0001$).

0.0001). ***$p < 0.001$, ****$p < 0.0001$ (n = 4). All transfections were performed with 50 nM of

negative control, siRNA controls, unmodified miR-129, or 5-FU-miR-129. Cells were also treated

with 450 nM of 5-FU to mimic the equivalent concentration of 5-FU in 5-FU-miR-129. Data are

represented as mean $\pm$ SD.

Figure 3: 5-FU-miR-129 Affects Autophagy in a Cell-Line Dependent Manner in NSCLC *in*

vitro. 4 hours prior to protein collection, cells were changed to serum-starved media (negative

control and 5-FU-miR-129) to induce autophagy or changed to fresh media + serum (negative

control + serum) to inhibit autophagy. (A) 5-FU-miR-129 did not have a significant effect on

autophagy in A549 cells, as observed by a non-significant change to LC3B-II/I ratio. ****$p < 0.0001$

(n = 5). (B) 5-FU-miR-129 was observed to promote autophagy in Calu-1 cells, as observed by a

significant increase in LC3B-II/I ratio ($p = 0.0255$). ***$p < 0.001$, ****$p < 0.0001$ (n = 3). All

transfections were performed with 50 nM of negative control and 5-FU-miR-129. Data are

represented as mean $\pm$ SD.

Figure 4: 5-FU-miR-129 Can Be Transfected Vehicle-Free to NSCLC *In Vitro*. (A) 5-FU-miR-

129 was found to be able knock down Bcl-2 ($p = 0.0012$) and HMGB1 ($p < 0.0001$) in A549 cells

without the aid of a transfection vehicle. **$p < 0.01$, ****$p < 0.0001$ (n = 3). (B) In addition, vehicle-

free transfection of 5-FU-miR-129 was found to inhibit cell proliferation, as seen by an 84.69%

reduction ($p < 0.0001$) in cell proliferation 6 days post-transfection via WST-1 assay. ****$p < 0.0001$

(n = 3). All transfections were performed with 50 nM of negative control, siRNA controls,

unmodified miR-129, or 5-FU-miR-129. Cells were also treated with 450 nM of 5-FU to mimic the

equivalent concentration of 5-FU in 5-FU-miR-129. Data are represented as mean $\pm$ SD.

Figure 5: 5-FU-miR-129 Can Overcome Resistance to Cancer Therapeutics in NSCLC *In*

Vitro. (A) The IC_{50} values of erlotinib and 5-FU-miR-129 were measured in an NSCLC cell line

that is resistant to erlotinib (HCC827ER). A 3.7-fold difference between the IC_{50} values of erlotinib

and 5-FU-miR-129 was observed (n = 3). [a]The IC_{50} of erlotinib in the parental line (HCC827) was

determined by previously reported values.[40,41] (B) 5-FU-miR-129 inhibits cell proliferation in

HCC827ER. 6 days post-transfection, a 90.03% reduction in cell proliferation was observed in

cells treated with 5-FU-miR-129 ($p < 0.0001$). All transfections were performed with 50 nM of

negative control, miR-129, or 5-FU-miR-129. *$p < 0.05$, ****$p < 0.0001$ (n = 4). (C) The IC_{50} values

of 5-FU and 5-FU-miR-129 were measured in an NSCLC cell line that is resistant to the

chemotherapy drug 5-FU (A549-5FU). An 837-fold difference between the IC_{50} values of 5-FU

and 5-FU-miR-129 was observed (n = 3). (D) 5-FU-miR-129 inhibits cell proliferation in A549-5FU

cells. 6 days post-transfection, a 75.56% reduction in cell proliferation was observed in cells

treated with 5-FU-miR-129 ($p = 0.0009$). All transfections were performed with 50 nM of negative

control, miR-129, or 5-FU-miR-129. A549-5FU cells were also treated with 5-FU at 450 nM or

11.67 μM to mimic the equivalent concentration of 5-FU in 5-FU-miR-129 or to mimic the observed

IC_{50} of 5-FU in A549 to demonstrate resistance to 5-FU. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ***$p <$

0.0001 (n = 3). Data are represented as mean $\pm$ SD.

Figure 6: 5-FU-miR-129 Affects Global mRNA Expression in Targets Related to Cell Death.

NSCLC cell lines A549 and Calu-1 were transfected with either a negative control or 5-FU-miR-

129. Extracted RNA per condition were pooled together (n = 3) and used in QIAGEN's RT^2 Profiler

Array to identify changes in mRNA expression in targets related to cell death via RT-PCR. (A, B)

Changes in mRNA expression were identified in A549 cells and the results were analyzed and

visualized for pathway analysis via GeneSpring GX and Enrichr. (C, D) Changes in mRNA

expression were also identified in Calu-1 cells and the results were analyzed and visualized for

pathway analysis via GeneSpring GX and Enrichr.

Figure 7: 5-FU-miR-129 Can Inhibit NSCLC Tumor Growth *In Vivo* and Promote Survival. A

luciferase-expressing line of NSCLC (A549-Luc) was injected (1 x 10^7 cells per mouse) into NOD-

SCID mice via intravenous tail vein injection. Post-tumor injection, mice were given two rounds of

treatment (D2-16 and D33-40) with either vehicle control (negative), 5-FU (50 mg/kg),

gemcitabine (Gem) (50 mg/kg), or 5-FU-miR-129 (3.75 mg/kg) via intravenous tail injection

(negative and 5-FU-miR-129) or intraperitoneal injection (IP) (5-FU and Gem). (A) Tumor growth

was monitored via luciferase expression using the IVIS system as seen by IVIS images of the

negative and the 5-FU-miR-129 treated group. IVIS images of 5-FU and Gem treated groups are

available in Figure S3. (B, C) 37 days post-injection of tumor cells, 5-FU-miR-129 (p = 0.0008)

and Gem (p = 0.0049) inhibited NSCLC tumor growth when compared to the negative control

group. **p< 0.01, ***p< 0.001 (n = 5). (D) Mice were also monitored for survival, and only mice

treated with 5-FU-miR-129 were found to have extended survival from 86 days to 100 days (HR

= 0.3209, p = 0.0391). Data are represented as mean ± SD.

A

5-FU-miR-129   5'-CUUUUGCGGUCUGGGCUUGC-3'
                    | | | | | | | | | |
BCL2 3' UTR   3'...AAAAAAACAGUCCAAAAGUUUAU...5'

B

C

D

A

HCC827	IC$_{50}$ (nM)
Erlotinib	6.5 – 22[a]
HCC827ER	**IC$_{50}$ (nM)**
Erlotinib	64.26
5-FU-miR-129	17.59

B

C

A549	IC$_{50}$ (nM)
5-FU	11,665
A549-5FU	**IC$_{50}$ (nM)**
5-FU	29,570
5-FU-miR-129	35.32

D

A

Day 37

Negative

5-FU-miR-129
(3.75 mg/kg)

Luminescence

1.0

0.8

0.6 $\times 10^6$

0.4

0.2

Radiance
(p/sec/cm²/sr)

Color Scale
Min = 1.00e5
Max = 1.00e6

B

Fold Change in Tumor Growth

Fold Change

n.s.

**

***

Negative
5-FU (50 mg/kg)
Gem (50 mg/kg)
5-FU-miR-129 (3.75 mg/kg)

C

Tumor Expression vs. Treatment

D2   D16   D33

Fold Change

n.s.

**

***

0   10   20   30   40

Day

- ● Negative
- ■ 5-FU (50 mg/kg)
- ▲ Gem (50 mg/kg)
- ▼ 5-FU-miR-129 (3.75 mg/kg)

D2: Treatment 1 Start (14 Days)
D16: Treatment 1 End
D33: Treatment 2 Start (7 Days)

D

Survival

Probability of Survival

0   50   100   150

Days Post Injection

- ● Negative
- ■ 5-FU (50 mg/kg)
- ▲ Gemcitabine (50 mg/kg)
- ▼ 5-FU-miR-129 (3.75 mg/kg)

* p = 0.0391