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1 **Development of 5-FU-modified tumor suppressor microRNAs as a platform for novel**
2 **microRNA-based cancer therapeutics**
3

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27 **Abstract**

28 MicroRNA (miRNAs) are pleiotropic post-transcriptional modulators of gene expression. Its
29 inherently pleiotropic nature make miRNAs strong candidates for the development of cancer
30 therapeutics, yet despite its potential, there remains a challenge to deliver nucleic acid-based
31 therapies into cancer cells. We developed a novel approach to modify miRNAs by replacing the
32 uracil bases with 5-fluorouracil (5-FU) in the guide strand of tumor suppressor miRNAs, thereby
33 combining the therapeutic effect of 5-FU with tumor suppressive effect of miRNAs to create a
34 potent, multi-targeted therapeutic molecule without altering its native RNA interference (RNAi)
35 function. To demonstrate the general applicability of this approach to other tumor suppressive
36 miRNAs, we screened a panel of 12 novel miRNA mimetics in several cancer types including
37 leukemia, breast, gastric, lung, and pancreatic cancer. Our results show that 5-FU-modified
38 miRNA mimetics have increased potency (low nM range) in inhibiting cancer cell proliferation
39 and that these mimetics can be delivered into cancer cells without delivery vehicle both *in vitro*
40 and *in vivo*, thus representing significant advancements in the development of therapeutic
41 miRNAs for cancer. This work demonstrates the potential of fluoropyrimidine modifications that
42 can be broadly applicable and may serve as a platform technology for future miRNA and nucleic
43 acid-based therapeutics.

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53 **Introduction**

54 MicroRNAs (miRNAs) are a class of short non-coding RNA that were discovered in 1993
55 and were observed to have critical regulatory functions, regulating protein biosynthesis via direct
56 interaction with the 3' untranslated region (3'-UTR) of target mRNA transcripts.[1,2] MiRNA are
57 key mediators of the RNA interference (RNAi) pathway and its interactions are largely governed
58 by its seed sequence on the 5' end of the miRNA. They are thus pleiotropic regulators of gene
59 expression, where one particular miRNA can interact with multiple mRNA target transcripts.
60 There are numerous studies demonstrating the critical roles of miRNAs in human disease and
61 miRNA expression is often dysregulated in cancer. Due to their impact on numerous
62 biochemical pathways, different miRNAs have been observed to either promote or inhibit
63 tumorigenesis and tumor growth in cell context-dependent manner. These miRNAs are referred
64 to as oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs respectively.[3] miR-15 was
65 the first miRNA to be found either absent or downregulated in cancer and it was subsequently
66 discovered that it modulates apoptosis by directly regulating BCL2 expression.[4,5] Since then,
67 numerous cancer types have been shown to have dysregulated tumor suppressor miRNA
68 expression. The functional significance of miRNAs in cancer has also been further studied, and
69 tumor suppressor miRNAs that regulate key oncogenic pathways—such as apoptosis,
70 proliferation, autophagy, cell cycle, and epithelial-to-mesenchymal (EMT) transition—have been
71 identified and studied.[6-13] Experimental evidence that demonstrates the key role of miRNAs in
72 these pathways reveals the therapeutic potential for tumor suppressor miRNAs.

73 There are, however, several hurdles need to be overcome to realize the therapeutic
74 potential of miRNAs. One of the major bottlenecks in the development of nucleic acid-based
75 medicine is the efficient intracellular delivery of these molecules. Tremendous effort in the past
76 several decades has been devoted to developing delivery vehicle technologies and various
77 lipid-based nanoparticles—including liposomes, micelles, and dendrimers—have grown in
78 popularity.[14,15] Many of these lipid nanoparticles (LNPs) function as cationic polymers that

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79 facilitate the transport of oligonucleotides across the plasma membrane. Despite the
80 advancements in lipid-based delivery systems, there are still barriers to its use. LNPs still face
81 several challenges such as their instability, rapid systemic clearance, and toxicities—including
82 their induction of immunostimulatory responses. In preclinical studies, LNPs are known to
83 exhibit some cytotoxicity in various cell lines and mice treated with some formulations of
84 positively-charged LNPs showed increased liver enzymes and body weight loss.[16,17] In humans,
85 anti-polyethylene glycol (PEG) antibodies are generated from pegylated drugs, including LNP
86 formulations, and PEG-induced complement activation has also been observed.[18-21] Toll-like
87 receptors (TLRs) are involved in the innate immune response and typically recognize molecular
88 patterns associated with pathogens.[22] TLR-4 activation has also been reported with the use
89 LNPs.[16] These toxicities are particularly problematic as they are often observed when LNPs are
90 delivered systemically, the main route of delivery in cancer therapy.[23-25]

91 Other approaches to enhance delivery include the modification of the nucleic acids
92 themselves. Modifications of the sugar-phosphate backbone and/or the base have been shown
93 to enhance delivery, stability, and potency of various nucleic acid-based therapeutics.[26] 5-
94 fluorouracil (5-FU) is a pyrimidine analog and is converted to fluorodeoxyuridylate (FdUMP) to
95 inhibit the sole *de novo* biosynthesis of thymidylate by the formation of suicide ternary complex
96 with its target enzyme protein thymidylate synthase (TS) along with tetrahydrofolate.[27] Due to
97 this antimetabolite effect, 5-FU has been used as a major chemotherapeutic agent for many
98 cancer types. 5-FU can be incorporated into the miRNA molecule and we have previously taken
99 this approach with three tumor suppressor miRNAs—miR-15a, miR-129, and miR-489—and
100 have demonstrated that 5-FU modification of these miRNAs are an effective and potent cancer
101 therapeutic in *in vivo* mouse models of colon, pancreatic, and breast cancer.[28-31] Additionally,
102 this modification confers increased intracellular stability of the miRNA, a novel ability to be
103 delivered into cells without use of delivery vehicle, and retention of target specificity. This
104 modification strategy greatly overcomes some of the bottlenecks of nucleic acid-based drug

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105 development by improving the deliverability of miRNAs as well enhancing its potency. To
106 demonstrate the general applicability of this concept, we took this unique 5-FU modification-
107 approach to develop a miRNA strategy that can be implemented as a platform for miRNA-based
108 cancer therapeutics.

109 In this study, we selected 12 well-studied tumor suppressor miRNAs (hsa-let-7a, hsa-
110 miR-15a, hsa-miR-34, hsa-miR-129, hsa-miR-140, hsa-miR-145, hsa-miR-194, hsa-miR-200a,
111 hsa-miR-200b, hsa-miR-200c, hsa-miR-215, and hsa-miR-506) as candidates to apply this
112 modification strategy.[6-13,32-37] These tumor suppressor miRNAs are often dysregulated in cancer
113 cells and play important roles in multiple pathways regulating the cell cycle, apoptosis, EMT,
114 metastasis, and drug resistance.[38] We substituted the uracil bases on the guide strand of the
115 miRNAs with 5-FU and screened these 5-FU-modified miRNA mimetics for efficacy in various
116 cancer types. The passenger strand of miRNA was left unmodified to avoid potential off-target
117 effects and to preserve the function of miRNA. Notably, 5-FU modification does not affect
118 Watson-Crick base-pairing, as the fluorine substituted for the hydrogen at the 5-carbon is not
119 involved in normal hydrogen bonding between nucleobases. This unique strategy combines the
120 therapeutic effect of 5-FU with the tumor suppressor function of miRNAs to inhibit multiple
121 oncogenic targets and pathways of cancer cells. Our results show that these 5-FU-modified
122 miRNA mimetics were able to reduce cancer cell proliferation with enhanced potency while
123 preserving target specificity. Lastly, the therapeutic effects were achieved without the aid of any
124 delivery vehicle both *in vitro* and *in vivo*.

125

126 **Results**

127 **Sequence and structure of the miRNA mimetics with 5-FU modification**

128 To evaluate 5-FU modification as a universal strategy for all tumor suppressor miRNAs and for
129 multiple major cancer types, we selected twelve tumor suppressor miRNAs that are well studied
130 in several cancer types, including in gastric cancer, lung cancer, breast cancer, leukemia, and

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131 pancreatic cancer: hsa-let-7a, hsa-miR-15a, hsa-miR-34, hsa-miR-129, hsa-miR-140, hsa-miR-
132 145, hsa-miR-194, hsa-miR-125, hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, and hsa-miR-
133 506 (herein after referred to without their hsa- prefix which refers to their human species of
134 origin). We designed a panel of 5-FU-modified miRNAs by substituting the uracil bases of the
135 guide strand of the mature miRNA with the antimetabolite nucleoside analog 5-FU **(Figure 1)**.
136

137 **Toxicity of Nucleic Acid Delivery Vehicle**

138 To demonstrate the cytotoxicity of delivery vehicles, both gastric cancer AGS and lung
139 cancer A549 cells were treated with a polyethyleneimine-based lipid nanoparticle (*in vivo*-
140 jetPEI®, Polyplus) and a dose-dependent increase in apoptosis was observed **(Figure 2)**. It is
141 clearly evidenced that while PEI has minimal impact on cell death at low concentrations, PEI is
142 cytotoxic at higher concentration and triggers apoptosis.
143

144 **5-FU-modified miRNA mimetics display enhanced efficacy at inhibiting cancer**
145 **proliferation and can enter the cell without the use of delivery vehicle**

146 To systematically confirm that vehicle-free delivery of the 5-FU-modified miRNA
147 mimetics is a reproducible, sequence-independent feature and that is also not dependent on the
148 number of 5-FU substitutions, we explored the efficacy of our panel of 5-FU-modified miRNAs in
149 different tumor types, we performed a screen by treating cells with 50 nM of the 5-FU-modified
150 miRNAs without the use of delivery vehicle in the following cell lines: AGS gastric cancer cells,
151 A549 lung cancer cells, SBKR3 breast cancer cells, REH leukemia cells, and AsPC-1, Hs766T,
152 and PANC-1 pancreatic cancer cells **(Figure 3)**. Our results clearly show that while unmodified,
153 control miRNAs have no effect due to the inability to cross the cell membrane, the 5-FU
154 modified miRNA mimetics are all showing efficacy to inhibit cancer cell proliferation **(Figure 3)**.
155 In addition to vehicle free delivery, there is enhanced therapeutic efficacy of 5-FU-
156 modified miRNA mimetics compared to their unmodified, native counterparts in both AGS cells

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157 **(Table 1)** and A549 cells **(Table 2)**. In AGS cells, there is an 8.4-fold increase in the inhibition of
158 cell proliferation after 5-FU modification of let-7a (let-7a $IC_{50}$ = 59.5 nM vs. 5-FU-let-7a $IC_{50}$ = 7.1
159 nM) **(Figure 4A)** and a 4.3-fold increase for miR-145 (miR-145 $IC_{50}$ = 20.9 nM vs. 5-FU-miR-145
160 $IC_{50}$ = 4.9 nM) **(Figure 4B)**. Similarly, in A549 cells, there is a 6.3-fold increase in the inhibition
161 of cell proliferation after modification of let-7a with 5-FU (let-7a $IC_{50}$ = 143.5 nM vs. 5-FU-let-7a
162 $IC_{50}$ = 22.8 nM) **(Figure 4C)** and a 13.8-fold increase for miR-145 (miR-145 $IC_{50}$ = 51.7 nM vs. 5-
163 FU-miR-145 $IC_{50}$ = 11.0 nM) **(Figure 4D)**.

164 To further confirm that miRNAs 5-FU-let-7a and 5-FU-miR-145 can enter the cell without
165 the use of transfection vehicle, AGS and A549 cells were treated let-7a and miR-145 without the
166 use of vehicle. There is no inhibition of cell proliferation in cells treated with let-7a and miR-145
167 without the use of vehicle **(Figure 4E-4H)** compared to cells treated with let-7a and miR-145 in
168 the presence of vehicle (**Figure 4A-4D)**. Notably, 5-FU-let-7a and 5-FU-miR-145 has a
169 significant inhibition of proliferation despite the absence of delivery vehicle **(Figure 4E-4H)**.

170 The main mechanism of action of 5-FU is forming an irreversible, covalent ternary
171 complex with thymidylate synthase (FdUMP-TS) and tetrahydrofolate. To investigate whether
172 the enhanced efficacy of 5-FU-modified miRNA mimetics are due to the release of 5-FU,
173 western immunoblot analysis of thymidylate synthase (TS) was used to detect the presence of
174 the FdUMP-TS complex. We probed for TS to evaluate whether the 5-FU-modified miRNA
175 mimetics exerted a 5-FU effect. Upon western immunoblot analysis, we observed that 5-FU-let-
176 7a and 5-FU-miR-145 exerts a 5-FU effect as observed by the formation of FdUMP-TS
177 complex, represented as the upper band, in both AGS cells **(Figure 5A & 5B)** and in A549 cells
178 **(Figure 5C & 5D)**.

179

180 **Target specificity of 5-FU-modified miRNA mimetics**

181 To confirm the target specificity of the 5-FU-modified miRNA mimetics, we performed
182 western blot analysis on known targets of let-7a and miR-145. Cyclin-dependent kinase 6

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183 (CDK6) is a direct target of let-7a.[39,40] Similarly, specificity protein 1 (SP1) is a direct target of
184 miR-145.[41,42] In both AGS gastric cancer cells and A549 lung cancer cells 5-FU-let-7a
185 decreases CDK6 expression **(Figure 6A & 6B)** and 5-FU-miR-145 decreases SP1 expression
186 under vehicle-free conditions **(Figure 6C & 6D)**. To further confirm that 5-FU-modified miRNA
187 mimetics are effective under vehicle-free conditions, we performed western blot analysis of
188 checkpoint kinase 1 (CHK1) and WEE1 G2 checkpoint kinase (WEE1), two direct targets of
189 miR-15a.[29] 5-FU-miR-15a decreases the expression of both CHK1 and WEE1, whereas neither
190 miR-15a, 5-FU, nor the combination of the two decreases target protein expression under
191 vehicle-free conditions **(Figure 6E).**

192

193 **Therapeutic efficacy and safety of 5-FU-modified miRNA mimetics *in vivo***

194 To demonstrate the therapeutic efficacy of 5-FU modified miRNA mimetics *in vivo*, with
195 and without the delivery vehicle, we choose 5-FU-miR-15a as our top candidate based on the *in*
196 *vitro* efficacy screening. The *in vivo* effects of a 5-FU-modified miRNA mimetic, 5-FU-miR-15a,
197 was evaluated in a CT-26 syngeneic mouse colon cancer model. Mice were treated with PEI
198 vehicle alone, 40 µg of 5-FU-miR-15a with PEI vehicle, or 40 µg of 5-FU-miR-15a without
199 vehicle. Based on histopathological analysis of the tumors in the mouse lungs, compared to
200 control mice, 5-FU-miR-15a inhibits tumor growth by 58.2% without vehicle ($p = 0.0066$) **(Figure**
201 **7B)** and by 97.2% **(Figure 7C)** with vehicle ($p = < 0.0001$). This tumor inhibition was enhanced
202 in the presence of vehicle ($p = 0.0179$) **(Figure 7D)**. To assess whether these animals exhibited
203 any acute toxicity associated with the treatment, their body mass was measured daily during the
204 treatment period. The body mass of the animals stayed within normal healthy limits (<10%
205 change in mass) throughout the treatment period **(Figure 7E)** and liver chemistries did not differ
206 between the treatment groups (**Figure 1**).

207

208 **Discussion**

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209 In this study, we aimed to design a strategy to develop effective miRNA-based
210 therapeutics with enhanced efficacy and stability that can potentially subvert the use of delivery
211 vehicle. One of the key barriers in the development of nucleic acid-based therapeutics is finding
212 methods to effectively introduce these molecules into cells in a minimally cytotoxic manner.
213 While there have been some recent successes with using lipid-based nanoparticles as a vehicle
214 to deliver nucleic-acids in some diseases, toxicities associated with systemic delivery, including
215 immunotoxicity, still exist.[43-47] The toxicity of delivery vehicle becomes more profound and
216 prohibitory during dose escalation studies as increasing amount of vehicle are needed to deliver
217 the respectively increasing nucleic-acid drug candidates. While delivery vehicles may be
218 relatively safe at low concentrations, this approach relies on an extremely potent biological
219 response to the nucleic acid drug candidate. This is the case in mRNA vaccines, where a small
220 amount of mRNA, and thereby its vehicle, is required for an immunological response. However,
221 in the situation where higher dose of vehicle are needed to pack proportionally larger amounts
222 of nucleic acids, toxicities will become a major bottleneck for safe and effective therapy. Among
223 the 14 currently FDA approved oligonucleotide-based therapeutics, only 6 are delivered
224 systemically. Notably, the use of patisiran (Onpattro) requires premedication consisting of a
225 corticosteroid, acetaminophen, a H1 blocker, and a H2 blocker to prevent infusion-elated
226 reactions.[25] In this study, we demonstrated an example of toxicities associated with lipid-based
227 nanoparticles, exhibiting a dose-dependent effect on apoptosis **(Figure 2)**.

228 As a result of these observed toxicities, we sought to enable the use of miRNA-based
229 therapeutics that may be able to get away from the use of large amounts of delivery vehicle or
230 eliminating their use altogether. In this study, we focused our efforts to demonstrate the broad
231 potential of miRNA-based therapeutics by enhancing its deliverability. This novel approach was
232 approached by modified guide strand of tumor suppressor miRNA by replacing the uracils with
233 5-FUs (**Figure 1**). This approach has minimal alterations of the miRNA molecule, as the single
234 hydrogen to fluorine substitution at the 5-position of uracil does not interfere with its Watson-

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235 Crick base pairing with adenine. By incorporation of 5-FU to miRNA, we were able to deliver the
236 5-FU modified miRNA mimetics under without use of delivery vehicle *in vitro* **(Figure 3)**. These
237 modifications also enhanced efficacy of native miRNA by combine the therapeutic effects of
238 miRNA and 5-FU into one entity, as well as creating a more stable molecule against
239 degradation.[30] We have previously demonstrated in colon cancer, pancreatic, and breast cancer
240 that 5-FU modification of miRNAs have several key features such as retaining target specificity,
241 vehicle-free delivery, enhanced potency, and increased intracellular stability.[28,30,31,48] To
242 demonstrate the general applicability of this approach to other tumor suppressor miRNAs, we
243 chose 12 well-studied tumor suppressor miRNAs and applied our 5-FU modification strategy.
244 We screened these 12 different 5-FU-modified miRNA mimetics in several other major cancer
245 types including breast cancer, gastric cancer, leukemia, lung cancer, and pancreatic cancer.
246 While it is impractical to screen all potential tumor suppressor miRNAs, the tumor suppressor
247 miRNAs selected in this study have been previously shown to inhibit cancer growth, disease
248 progression, metastasis, and/or drug resistance.[38] Their biological roles and their targets are
249 also defined, well-investigated, and briefly summarized below. As previously stated, miR-15
250 regulates apoptosis by targeting BCL2. Similarly, miR-129 can also regulate BCL2 expression
251 and other protumorigenic proteins such as high-motility group box-1 (HMGB-1) and CDK6.[7,49,50]
252 The let-7 family regulates the RAS oncogene expression and is found to be downregulated in
253 multiple cancer types.[6,51,52] miR-34 directly targets P53 and thus it has an impact on multiple
254 pathways including tumor proliferation, apoptosis, and cell cycle. [35,53-57] miR-140 regulates
255 stemness through some of its targets HDAC4, SOX9, and ALDH1. miR-145 expression is found
256 to be highly downregulated in colon cancer[9] and regulates cancer growth, in several tumor
257 types, through its targets that include IGF1R, MYO6, and SP1.[9,41,58-62] miR-194 inhibits
258 metastasis and invasion by inhibiting BMP1, p27$^{kip1}$, and RBX1.[63-65] The miR-200 family
259 (including miR-200a/b/c) has been shown to inhibit EMT by downregulating ZEB1 and ZEB2 in
260 multiple tumor types including breast, gastric, lung, and pancreatic cancer.[8,36,66-69] miR-215 is a

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261 cell cycle regulator and delivering miR-215 to cancer cells causes cell cycle arrest in several
262 cancer types.[11,70,71] Lastly, miR-506 also regulates cancer progression and invasion by targeting
263 the NF-κB pathway, SNAI1, and YAP1.[33,34,72,73] In summary, these tumor suppressor miRNAs
264 have diverse and sometimes context-dependent biological functions thus it is important to
265 screen multiple therapeutic candidates.

266 During this screen, we observed that all 12 5-FU-modified miRNA mimetics were able to
267 inhibit cancer cell proliferation at a concentration of 50 nM without use of delivery vehicle
268 (**Figure 3**). Although we noticed that each unique 5-FU modified miRNA mimetic has different
269 levels of efficacy *in vitro*, interestingly, certain cell lines—AGS gastric cancer cells and REH
270 leukemia cells—were highly sensitive to all 5-FU-modified-miRNA-mimetic treatment **(Figure 3A
271 &3D)**. This raises an interesting finding that for gastric cancer and leukemia, 5-FU-modified
272 miRNA mimetics are all strong potential therapeutic candidates despite the different targets and
273 pathways that are impacted by each miRNA. Although we have demonstrated previously that 5-
274 FU modification of miRNAs (miR-129, miR-15a, miR-489) does not alter target specificity[28-31], to
275 further validate that target specificity is maintained with this approach, we selected two
276 additional miRNAs to investigate further—let-7a and miR-145—as they are previously reported
277 to be markers of aggression and prognosticators in gastric cancer[41,42,60,74-76] and in lung
278 cancer[58,59,61,77,78]. Our results demonstrate that 5-FU-let-7a and 5-FU-miR-145 retain target
279 specificity to CDK6 and SP1 respectively, indicating that there is successful knockdown of their
280 previously reported target genes **(Figure 6A-6D)**. To further validate that 5-FU-modified miRNA
281 mimetics retain target specificity and can do so under vehicle-free conditions, we investigated
282 two direct targets of miR-15a, CHK1 and WEE1. Our results demonstrated that 5-FU-miR-15a
283 can knock down target expression, without delivery vehicle, while neither miR-15a, 5-FU, nor
284 the combination of two are able to knock down expression **(Figure 6E)**. Collectively, our results
285 demonstrated that 5-FU modification of tumor suppressor miRNAs retain target specificity. We
286 also observe that 5-FU-let-7a and 5-FU-miR-145 form a FdUMP-TS complex, demonstrating

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287 that 5-FU is indeed released, potentially as a breakdown product of these mimetics, and can
288 exert 5-FU activity in cells **(Figure 5)**. Taken together, our results show that 5-FU-modified
289 miRNA mimetics are potent therapeutic molecules, effective in the nanomolar range and that
290 they are more effective at inhibiting cancer cell proliferation than unmodified miRNAs.

291 To demonstrate the therapeutic potential of 5-FU modified miRNA mimetics *in vivo*, we
292 selected 5-FU-miR-15a based on our *in vitro* screening using an immunocompetent syngenetic
293 mouse colon cancer model. Using a tail vein injection syngenetic colon cancer mouse model,
294 we observed that 5-FU-miR-15a can inhibit cancer cell growth and lung metastasis both with
295 and without delivery vehicle **(Figure 7A-7D)**. This model was selected as a proof-of-concept to
296 model efficacy in a metastatic disease setting, the largest burden on cancer morbidity and
297 mortality. It is worth noting that the therapeutic efficacy of 5-FU-miR-15a can be further
298 enhanced with a low concentration of a polyethyleneimine LNP delivery vehicle. This is an
299 expected outcome of this investigation, as we sought to create miRNA-based therapeutics that
300 relies only on a little to no delivery vehicle, key to avoiding potential toxicities **(Figure 7).** This is
301 the first time, to our best knowledge, miRNA-based cancer therapeutics have been
302 demonstrated to be effective without the aid of delivery vehicle *in vivo*. Mice treated with 5-FU-
303 miR-15a with and without delivery vehicle show no significant weight and hair loss **(Figure 7E)**.
304 Liver chemistries also did not differ between treatment groups, potentially representing a lack of
305 hepatotoxicity from 5-FU-modified miRNA mimetic treatment **(Figure S1)**. Additionally, there
306 were also no behavioral changes, such as loss of appetite, among the treated mice. The
307 demonstration of efficacy in the presence and absence of delivery vehicle may allow for
308 flexibility in the optimization of the formulation of the mimetics for future clinical use, and to
309 avoid the bottleneck of toxicity. Future therapeutic development can consider the reduction or
310 elimination of vehicle thus potentially avoiding side effects due to toxicity as seen in systemic
311 chemotherapeutic regimens. Similarly, low toxicities may give rise to a larger therapeutic

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312 window of 5-FU-modified miRNA mimetics and further work must be completed to optimize the
313 dosage of these mimetics.

314 Previous studies from our group have compared a few different modification strategies of
315 miRNAs, including varying the number of 5-FU substitutions and locations of the moiety and
316 have demonstrated that the substation of all uracil bases on the guide strand appear to be the
317 most effective.[30] A potential mechanism of action of the observed enhanced efficacy and
318 deliverability of the 5-FU-modified miRNA mimetics is due to the increased lipophilicity that is
319 conferred with the addition of fluorine to drug candidates, which is a strategy that has been used
320 for improving lipophilicity of small molecule compounds.[79,80] This increase in lipophilicity may
321 allow for the miRNA—negatively charged and typically unable to cross the cell membrane—to
322 cross the cell membrane. Our approach takes advantage of 5-FU as an active anti-cancer
323 therapeutic compound and the fluorine group will also enhance the deliverability of a nucleic-
324 acid based miRNA tumor suppressor. There are various modification strategies in nucleic acid
325 drug development—especially in antisense oligonucleotides—including 2'-*O*-methyl that confers
326 more target affinity and 2'-fluoro that confers more nuclease resistance.[81,82] We have previously
327 observed that 5-FU modification in miR-129 confers additional intracellular stability[30], however in
328 this study, we did not observe an increase in half-life of 5-FU modified miRNA mimetics in cell
329 culture media supplemented with 10% FBS (data not shown). Notably, we did not make any
330 additional modifications to attempt to preserve the native miRNA tumor suppressor function.
331 Future studies can consider some of these approaches to optimize the design of these drug
332 candidates. It is also difficult to tease apart the individual contributions to the anti-tumor
333 phenotype of the 5-FU and the tumor suppressor miRNAs that make up the 5-FU-modified
334 miRNA mimetics. Future studies evaluating 5-FU-modified anti-miRNAs may help begin
335 answering some of those mechanisms. Similarly, while the 5-FU-modified miRNA mimetics
336 appear to be broadly effective, the selection of specific 5-FU-modified miRNA mimetics for

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337 further pre-clinical development should consider miRNA targets of oncogenic signaling
338 pathways and the specific cancer type.

339 In summary, our study demonstrates that 5-FU-modified miRNA mimetics display broad
340 therapeutic potential, as they are effective in several different cancer types without the aid of
341 transfection vehicle. This study expands on previous work on 5-FU-modified miRNA mimetics,
342 showing that they are efficacious in additional cancer types including gastric cancer, lung
343 cancer, and leukemia. Notably, 5-FU-modified miRNA mimetics retain their mRNA target
344 specificity and appears to be well tolerated in our animal studies. Overall, 5-FU modification of
345 miRNAs may serve as a novel technology platform for broad miRNA-based therapeutic
346 development.

347

348 **Materials and Methods**

349 **Design and synthesis of the 5-FU-modified miRNA mimetics**

350 The 5-FU-modified miRNA mimetics were designed and synthesized by substituting uracil with
351 5-fluorouracil (5-FU) on the guide strand of the miRNA. The passenger strand was left
352 unmodified to avoid any potential off-target effects and to preserve miRNA function.
353 Oligonucleotides with these modifications as well as their corresponding passenger strand were
354 purchased from Dharmacon (Horizon Discovery). Both strands of oligonucleotides were HPLC
355 purified. The guide strands and passenger strands were then annealed prior to use.

356

357 **Cell culture**

358 All cell lines were obtained from ATCC and are derived from human cells. AGS gastric cancer
359 and A549 lung cancer cells were cultured in Ham's F-12K (Kaighn's) Medium supplemented
360 with 10% fetal bovine serum (FBS). SKBR3 breast cancer cells and HCT116 colon cancer cells
361 were cultured in McCoy's 5A Medium supplemented with 10% FBS. REH leukemia cells and
362 AsPC-1 pancreatic cancer cells were cultured in RPMI-1640 Medium supplemented with 10%

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363 FBS. Hs766T and PANC-1 pancreatic cancer cells were cultured in Dulbecco's Modified Eagle
364 Medium (DMEM) supplemented with 10% FBS.
365

366 **Western immunoblot analysis**

367 AGS, A549, and HCT116 cells were seeded onto 6-well plates at a cell density of 100,000 cells
368 per well. 24 hours later, the cells were transfected with 50 nM of miRNA under vehicle-free
369 conditions. HCT116 cells were also treated with a 50 nM miR-15a control condiditon, a 350 nM
370 of 5-FU (equivalent 5-FU concentration in the 5-FU modified miRNA), and the combination of
371 the two. 72 hours later, cells were lysed with RIPA buffer and the protein samples were used for
372 western immunoblotting. Proteins were probed with anti-thymidylate synthase antibody
373 (Millipore, Cat:MAB4130 , 1:500), anti-CDK6 antibody (Cell Signalling, Cat:13331, 1:10,000),
374 anti-SP1 antibody (Abcam, Cat:ab124804, 1:10,000), β-actin antibody (Invitrogen, Cat:A5441,
375 1:10,000,000), anti-CHK1 antibody (Cell Signalling, Cat: ,1:1000), anti-WEE1 antibody (Cell
376 Signalling, Cat: , 1:1000), and anti-GAPDH antibody (Santa Cruz, Cat:, 1:100,000). Protein
377 bands were visualized using a LI-COR Biosciences Odyssey FC imaging system after the
378 addition of SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific).
379 Proteins were quantified with Image Studio Version 5.2.4 (LI-COR Biosciences). For the 5-FU
380 condition of the thymidylate synthase blot, AGS cells were treated with 1 μM of 5-FU and A549
381 cells were treated with 3 μM of 5-FU.
382

383 **Apoptosis assay**

384 AGS and A549 cells were treated with a polyethyleneimine-based lipid nanoparticle (*in vivo*-
385 jetPEI®, Polyplus). 48 hours later, the cells were stained with Annexin V (Thermo Fisher
386 Scientific) and apoptotic cells were quantified by flow cytometric analysis.
387

388 **Cell proliferation assay**

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389 For vehicle-free miRNA treatment, cells were seeded onto 96 well plates at 1000 cells per well.
390 24 hours after seeding, 5-FU-modified miRNA mimetics were added to the cells. These cells
391 were incubated for 24 hours and then the media was changed to fresh media supplemented
392 with 10% dialzed FBS. For miRNA treatement with vehicle, cells were seeded onto 6 well plates
393 at 100,000 cells per well. MiRNAs were combined with Oligofectamine™ (Thermo Fisher
394 Scientific) and then added to the cells. 24 hours later, the cells were trypsized and re-seeded
395 onto a 96 well plate at 1000 cells per well. Cell viability was measured 6 days post transfection
396 using WST-1 reagent (Roche). Cells were incubated with 10 µl of WST-1 per 100 µl of media for
397 1 hour and absorbance was read at 450 and 630 nm. The O.D. was calculated by subtracting
398 the absorbance at 630 nm from that at 450 nm and the relative proliferation was calculated by
399 normalizing the O.D. to negative control.
400
401 **Syngeneic mouse model**
402 8-week-old female BALB/cJ mice (Jackson Labs 000651) were inoculated with 5 x $10^5$ CT-26
403 syngeneic colon cancer cells suspended in 0.1 mL of PBS via tail vein injection. Five days post
404 inoculation, mice were treated with either 9.6 µM of vehicle alone (*in vivo*-jetPEI®, Polyplus), 5-
405 FU-miR-15a miRNA (40 µg) with vehicle, or 5-FU-miR-15a (40 µg) without vehicle on
406 alternating days for a total of 8 doses, with 5 mice per treatment group. Vehicle concentrations
407 were selected in a non-toxic range as per manufacturer recommendations. All treatments were
408 diluted in 5% glucose to a final volume of 0.1 mL and given via tail vein injection. Mouse tumors
409 were harvested from the lungs at the day 20 endpoint of the study and tumors were formalin
410 fixed, paraffin embedded, and mounted onto slides for staining with H&E. Slides were assessed
411 by a board certified pathologist and scored for % tumor content. Blood was also harvested at
412 the endpoint and was sent to a clinical chemistry lab for subsequent liver enzyme analysis.
413
414 **Statistical analysis**

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415 The quantitative data were presented as mean value ± standard error of the mean of at least 3
416 independent experiments in all in vitro studies. Data were analyzed by two-tailed Student's t-
417 test. The results of the animal studies presented as mean value ± standard deviation. A p value
418 of less than 0.05 were considered to be statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p <$
419 $0.001$, ****$p < 0.0001$).

420

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425

426 **Conflicts of Interest**

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

429

430 **Author Contributions**

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

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690 **Figure Legends**

691 **Figure 1. Panel of 5-fluorouracil-modified miRNAs.** The sequences of twelve tumor
692 suppressor miRNAs—let-7a, miR-15a, miR-34, miR-129, miR-140, miR-145, miR-194, miR-125,
693 miR-200a/b/c, and miR-506—with their uracil bases of the 5' strand of the mature miRNA
694 substituted with the antimetabolite nucleoside analog 5-fluorouracil (5-FU).

695

696 **Figure 2. Nucleic acid delivery vehicles are inherently toxic.** Common cationic lipid-based
697 nucleic acid delivery vehicles such as polyethyleneimine (PEI) are toxic to cells. **(A)** AGS and
698 **(B)** A549 cells exhibit dose-dependent apoptosis in the presence of PEI.

699

700 **Figure 3. Vehicle-free treatment of 5-FU-modified miRNAs exhibit potent inhibition of**
701 **tumor cell proliferation.** A panel of twelve 5-FU-modified miRNA mimetics were screened for
702 their effectiveness at inhibiting cell viability compared to negative control (scramble) miRNA at
703 50 nM without delivery vehicle. Several types of cancer cell lines were screened: **(A)** AGS
704 gastric cancer cells, **(B)** A549 lung cancer cells, **(C)** SBKR3 breast cancer cells, **(D)** REH
705 leukemia cells, and **(E)** AsPC-1, **(F)** Hs766T, and **(G)** PANC-1 pancreatic cancer cells.

706

707 **Figure 4. 5-FU-modification enhances the tumor suppressor effect of miRNAs and 5-FU-**
708 **modified miRNA mimetics can enter cells without delivery vehicle.** 5-FU-modified miR-145
709 (5-FU-miR-145) and 5-FU-modified let-7a (5-FU-let7a) inhibits cell proliferation both with and
710 without delivery vehicle in AGS gastric cancer cell line **(A-D)** and in A549 non-small cell lung
711 cancer cell line **(E-H).** Notably, 5-FU-modified miRNA mimetics appear to be able enter the cell
712 and inhibit cell growth, while unmodified miR-145 and let-7a are only able to inhibit tumor growth
713 in the presence of delivery vehicle. Data are presented as mean ± standard error of the mean
714 and analyzed by Student's t-test (n = 3).

715

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716 **Figure 5. 5-FU-let-7a and 5-FU-mi5-145 exhibits 5-FU activity. (A)** 5-FU-let-7a and **(B)** 5-FU-
717 miR-145 forms a TS-FdUMP ternary complex in AGS gastric cancer cells and A549 lung cancer
718 cells as seen by a band shift upon Western blot of TS. All cells were treated with 50nM of
719 miRNA and the 1μM of 5-FU in AGS cells and 3μM of 5-FU in A549 cells.
720

721 **Figure 6. 5-FU-modified miRNA mimetics retain target specificity.** CDK6 is a known target
722 of let-7a and 5-FU-let-7a can knockdown CDK6 expression in both **(A)** AGS gastric cancer cells
723 and **(B)** A549 lung cancer cells. SP1 is a known target of miR-145 and 5-FU-miR-145 knocks
724 down SP1 expression in both **(C)** AGS gastric cancer cells and **(D)** A549 lung cancer cells
725 under vehicle-free conditions. All cells were treated with 50 nM of either control scramble
726 miRNA or the respective miRNA. **(E)** 5-FU-miR-15a knocks down expression of its targets,
727 CHK1 and WEE1, under vehicle free conditions, whereas miR-15a, 5-FU, or the combination of
728 the two does not in HCT116 colon cancer cells. Data are presented as mean ± standard error of
729 the mean and analyzed by Student's t-test (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.
730

731 **Figure 7. 5-FU-miR-15a inhibits tumor growth in an *in vivo syngeneic* colorectal cancer**
732 **mouse model with and without delivery vehicle.** CT-26 tumor allografts established in 8-
733 week-old female BALB/cJ mice via tail vein injection following treatment (40 μg q.o.d.,8 doses)**.**
734 **(a-c)** Representative histological sections of tumors harvested after 20 days and stained with
735 H&E. (**d)** 5-FU-miR-15a inhibits tumor growth with and without PEI, though the efficacy is
736 improved with delivery vehicle ($p$ = 0.0179). **(e)** Body weight change was measured as an
737 indicator acute toxicity and no toxicity was observed (< 10% weight loss). Data are presented as
738 mean ± standard deviation and analyzed by Student's t-test (n = 5). *$p < 0.05$, **$p < 0.01$, ****$p$
739 $< 0.0001$.
740

741

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742

| Condition | $IC_{50}$ (nM) |
|---|---|
| **let-7a** | 59.5 |
| **5-FU-let-7a** | 7.1 |
| **miR-145** | 20.9 |
| **5-FU-145** | 4.9 |
| **5-FU** | 1745.7 |

743

744 **Table 1. $IC_{50}$ values of miRNAs in AGS cells.**

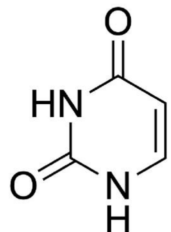
745

746

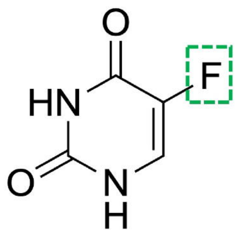
| Condition | $IC_{50}$ (nM) |
|---|---|
| **let-7a** | 143.5 |
| **5-FU-let-7a** | 22.8 |
| **miR-145** | 151.8 |
| **5-FU-145** | 11.0 |
| **5-FU** | 4612.0 |

747

748 **Table 2. $IC_{50}$ values of miRNAs in A549 cells.**




U Uracil




U 5-Fluorouracil

**5-FU-let-7a**

**5-FU-miR-15a**

**5-FU-miR-34**

**5-FU-miR-129**

**5-FU-miR-140**

**5-FU-miR-145**

**5-FU-miR-194**

**5-FU-miR-200a**

**5-FU-miR-200b**

**5-FU-miR-200c**

**5-FU-miR-215**

**5-FU-miR-506**

A

AGS

Apoptotic Cells (%)

100
75
50
25
0

0 25 50 75 100

PEI (μM)

B

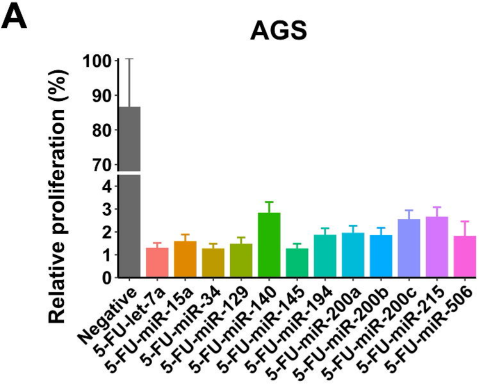
A549

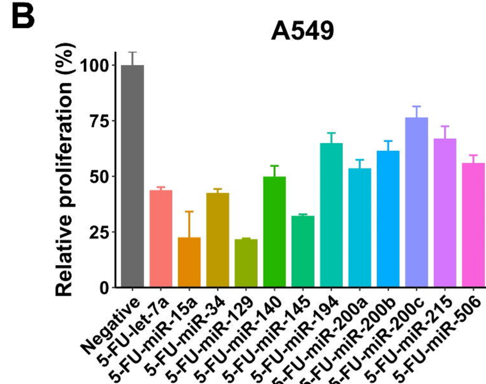
Apoptotic Cells (%)

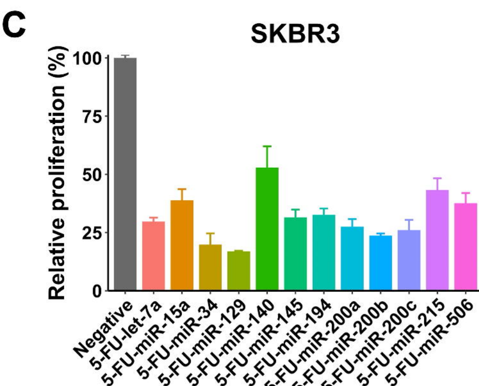
100
75
50
25
0

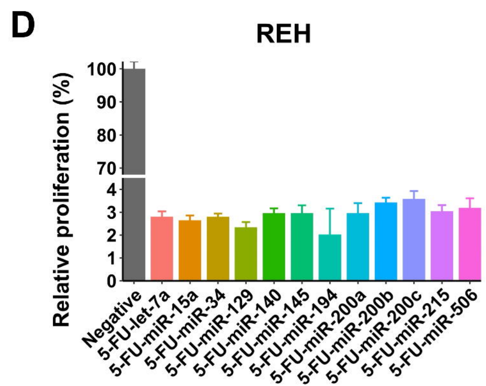
0 25 50 75 100

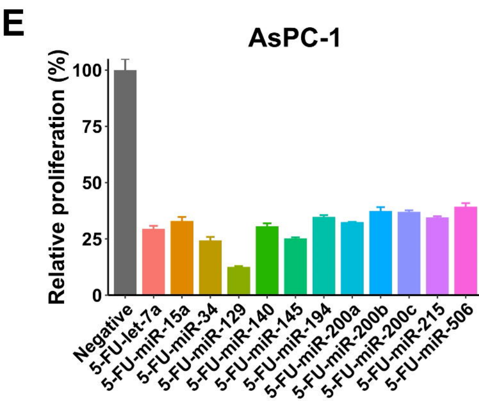
PEI (μM)

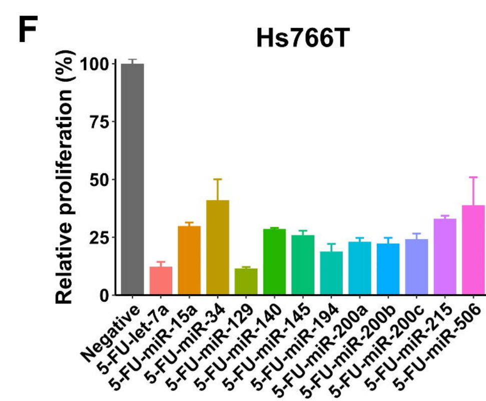

A
AGS
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

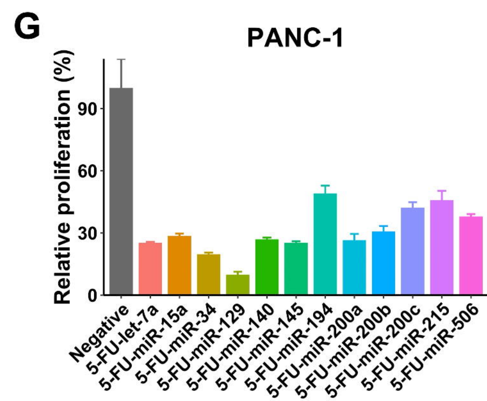

B
A549
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

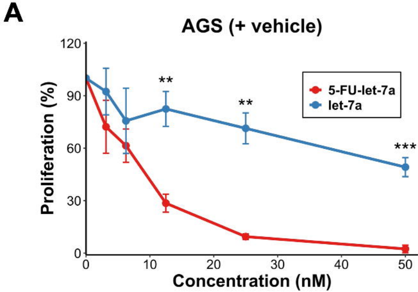

C
SKBR3
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

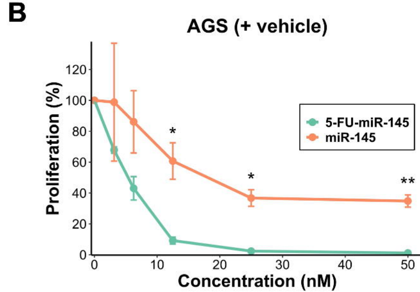

D
REH
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

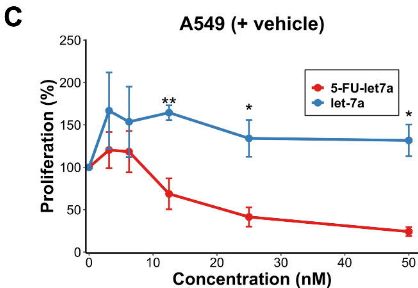

E
AsPC-1
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

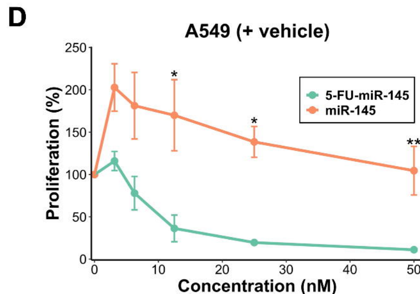

F
Hs766T
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

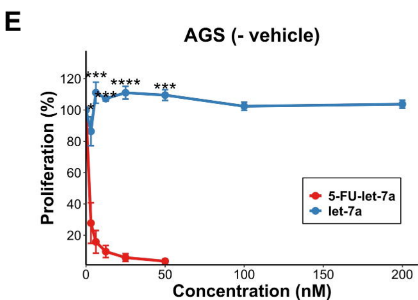

G
PANC-1
Relative proliferation (%)
Negative
5-FU-let-7a
5-FU-miR-15a
5-FU-miR-34
5-FU-miR-129
5-FU-miR-140
5-FU-miR-145
5-FU-miR-194
5-FU-miR-200a
5-FU-miR-200b
5-FU-miR-200c
5-FU-miR-215
5-FU-miR-506

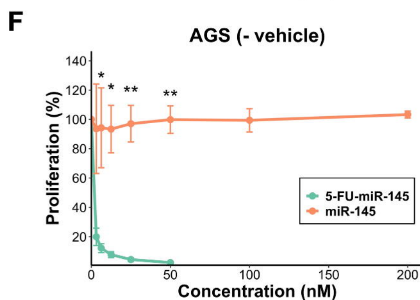

A
AGS (+ vehicle)
5-FU-let-7a
let-7a
Proliferation (%)
Concentration (nM)
**
**
***

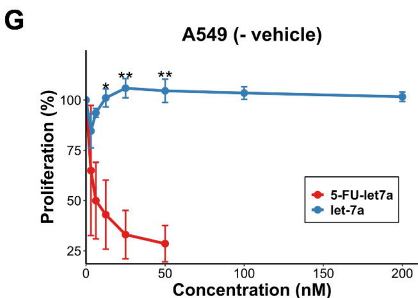

B
AGS (+ vehicle)
5-FU-miR-145
miR-145
Proliferation (%)
Concentration (nM)
*
*
**

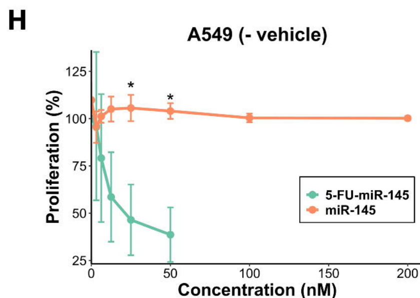

C
A549 (+ vehicle)
5-FU-let7a
let-7a
Proliferation (%)
Concentration (nM)
**
*
*

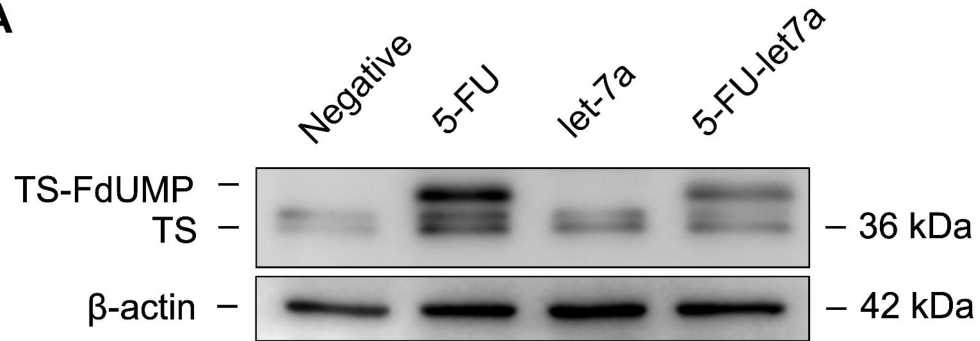

D
A549 (+ vehicle)
5-FU-miR-145
miR-145
Proliferation (%)
Concentration (nM)
*
*
**

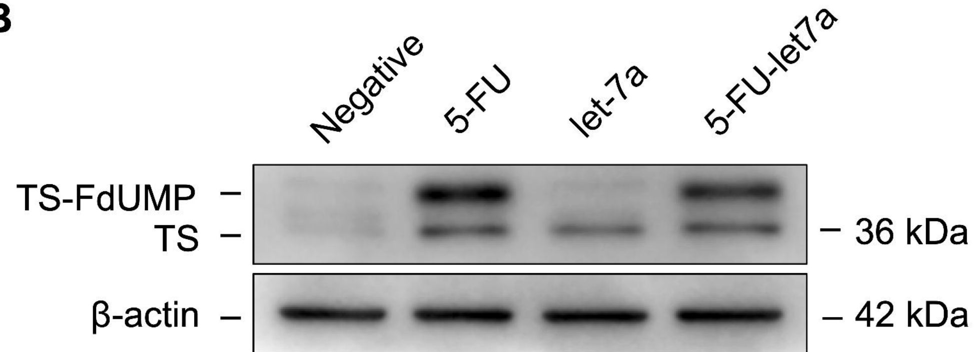

E
AGS (- vehicle)
5-FU-let-7a
let-7a
Proliferation (%)
Concentration (nM)
***
***
****
***
*

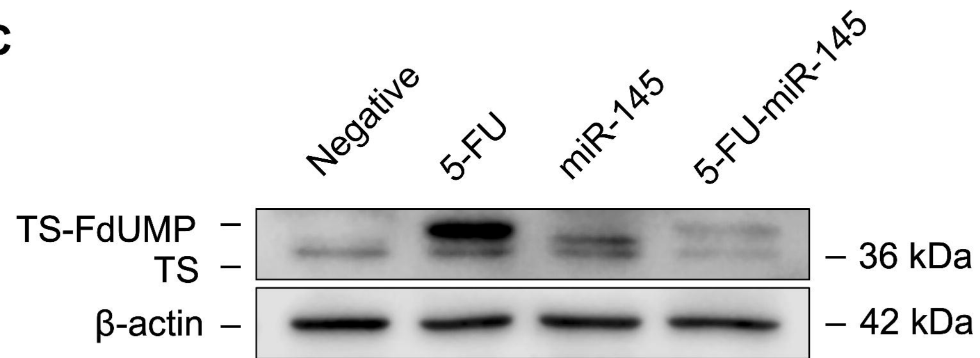

F
AGS (- vehicle)
5-FU-miR-145
miR-145
Proliferation (%)
Concentration (nM)
*
*
**
**

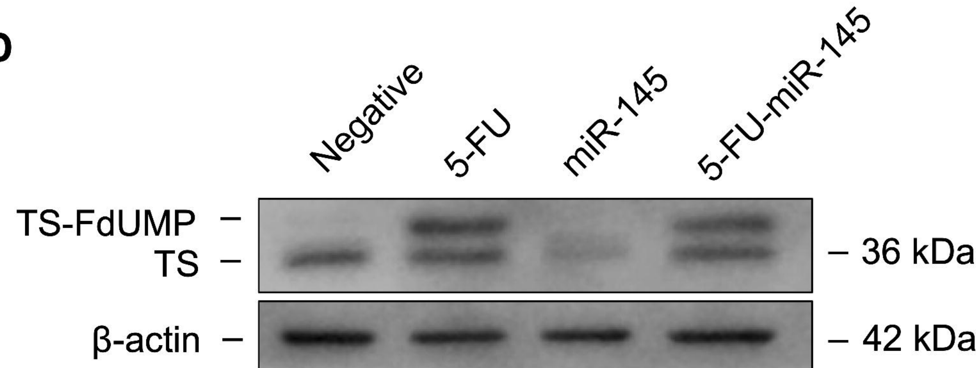

G
A549 (- vehicle)
5-FU-let7a
let-7a
Proliferation (%)
Concentration (nM)
*
**
**

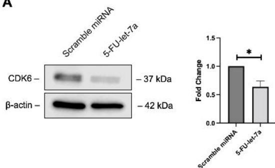

H
A549 (- vehicle)
5-FU-miR-145
miR-145
Proliferation (%)
Concentration (nM)
*
*

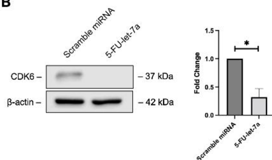

A
Negative
5-FU
let-7a
5-FU-let7a
TS-FdUMP
TS
β-actin
36 kDa
42 kDa

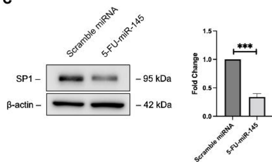

B
Negative
5-FU
let-7a
5-FU-let7a
TS-FdUMP
TS
β-actin
36 kDa
42 kDa

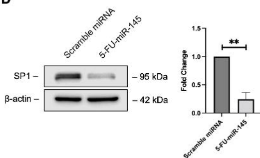

C
Negative
5-FU
miR-145
5-FU-miR-145
TS-FdUMP
TS
β-actin
36 kDa
42 kDa

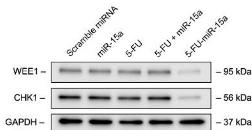

D
Negative
5-FU
miR-145
5-FU-miR-145
TS-FdUMP
TS
β-actin
36 kDa
42 kDa

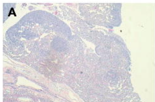

A
Scramble miRNA
5-FU-let-7a
CDK6 –
– 37 kDa
β-actin –
– 42 kDa
Fold Change
1.5
1.0
0.5
0.0
*
Scramble miRNA
5-FU-let-7a

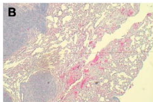

B
Scramble miRNA
5-FU-let-7a
CDK6 –
– 37 kDa
β-actin –
– 42 kDa
Fold Change
1.5
1.0
0.5
0.0
*
Scramble miRNA
5-FU-let-7a

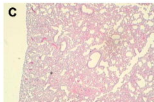

C
Scramble miRNA
5-FU-miR-145
SP1 –
– 95 kDa
β-actin –
– 42 kDa
Fold Change
1.5
1.0
0.5
0.0
***
Scramble miRNA
5-FU-miR-145

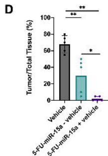

D
Scramble miRNA
5-FU-miR-145
SP1 –
– 95 kDa
β-actin –
– 42 kDa
Fold Change
1.5
1.0
0.5
0.0
**
Scramble miRNA
5-FU-miR-145

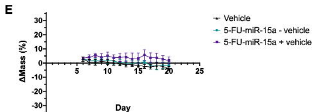

E
Scramble miRNA
miR-15a
5-FU
5-FU + miR-15a
5-FU-miR-15a
WEE1 –
– 95 kDa
CHK1 –
– 56 kDa
GAPDH –
– 37 kDa


A
Vehicle


B
5-FU-miR-15a - vehicle


C
5-FU-miR-15a + vehicle


D
Tumor/Total Tissue (%)
100
80
60
40
20
0
**
**
*
Vehicle
5-FU-miR-15a - vehicle
5-FU-miR-15a + vehicle


E
ΔMass (%)
30
20
10
0
-10
-20
-30
5
10
15
20
25
Day
Vehicle
5-FU-miR-15a - vehicle
5-FU-miR-15a + vehicle