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ORIGINAL ARTICLE

A combinatorial microRNA therapeutics approach to suppressing non-small cell lung cancer

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Targeted cancer therapies, although often effective, have limited utility owing to preexisting primary or acquired secondary resistance. Consequently, agents are sometimes used in combination to simultaneously affect multiple targets. MicroRNA mimics are excellent therapeutic candidates because of their ability to repress multiple oncogenic pathways at once. Here we treated the aggressive *Kras;p53* non-small cell lung cancer mouse model and demonstrated efficacy with a combination of two tumor-suppressive microRNAs (miRNAs). Systemic nanodelivery of miR-34 and *let-7* suppressed tumor growth leading to survival advantage. This combinatorial miRNA therapeutic approach engages numerous components of tumor cell-addictive pathways and highlights the ability to deliver multiple miRNAs in a safe and effective manner to target lung tissue.

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INTRODUCTION

Non-small cell lung cancer (NSCLC) is essentially untreatable, likely owing to accumulation of mutations that affect the RAS and p53 pathways, and is the leading cause of cancer-related deaths worldwide. Various strategies have been proposed to increase the survival of NSCLC patients including prevention, early diagnosis and more aggressive interventions for late-stage cancers. Indeed, 5-year survival rates can approach 70% with surgical resection of stage IA disease.[1] However, most individuals are diagnosed at late stage (at initial diagnosis, 55% of patients have distant metastatic disease) when their tumors and metastatic lesions have become refractory to current treatment regiments. As such, better treatments for late-stage, aggressive disease that do not solely depend on inhibiting a single biological target are needed.

Superior, targeted treatment options depend on the individual cancer genotype and the availability of tests to identify mutations susceptible to these therapies. NSCLC gene signatures have uncovered typical protein-coding gene mutations as well as subsets of microRNAs (miRNAs) that are aberrantly expressed.[2,3] Remarkably, the levels of two tumor-suppressive miRNAs, *let-7* and miR-34, are often the most statistically altered miRNAs in NSCLC tumor tissue.[2,4–8] The reduction in *let-7* and miR-34 expression is particularly relevant to the NSCLC oncogenic phenotype, as these miRNAs target key oncogenes involved in multiple stages of the tumorigenic process and in the maintenance of oncogene addiction, such as *RAS, BCL2, MET* and *MYC*.[4,6,9–12] In addition, miR-34 is a direct transcriptional target and produces phenotypes akin to p53.[12–16]

The recent discovery that miRNAs are modulators of key signaling pathways often dysregulated in disease has resulted in their emergence as powerful therapeutic agents, actively being evaluated for the treatment of multiple diseases (see Kasinski and Slack for a review[17]). These small non-coding RNAs efficiently modulate the expression of protein-coding genes either through translational repression or target messenger RNA destabilization.[18,19] Because miRNAs bind their targets with imperfect sequence complementarity, an individual miRNA is capable of affecting the expression of multiple genes. As such, the delivery of a single miRNA is analogous to a multidrug cocktail. Likewise, multiple miRNA binding sites are regularly found in an individual target gene, decreasing the likelihood of acquired resistance due to somatic mutations. Although the effect of an individual miRNA acting on a single target may be subtle, the collective repression of tens to hundreds of genes can have a significant impact on cells and produce strong phenotypic outcomes. This has been confirmed for tumor-suppressive miR-34 and its respective target genes *BCL2*, *MET*, *CDK4* and *c-MYC,* as well as for *let-7* and its targets *KRAS*, *c-MYC*, *HMGA2* and the *LIN28* isoforms.[4,6,9,11,12,20] Whereas the expression of miRNA target genes can vary in different tissues and cells; the ability of an miRNA to target multiple key oncogenes makes miRNAs an attractive therapeutic tool that is potentially more powerful than agents that target a single gene.

Both *let-7* and miR-34 function as tumor suppressors in NSCLC and can inhibit tumor growth in a variety of model systems when used therapeutically as single agents. Specifically, our groups and others have shown that exogenous *let-7* can both prevent and treat *Kras*-driven lung tumors,[21–23] and that miR-34 can prevent initiation and progression of $Kras^{G12D+/};p53^{R172H/+}$ lung tumors and human NSCLC tumor xenografts.[24–26] Additional studies showed that miRNAs are effective therapeutically even if they do not directly repress the mutant driver gene responsible for oncogenesis. Evidence comes from *Kras*-activated tumors that are exposed to exogenous miR-34;[25,26] although miR-34 is not expected to directly regulate *Kras*, there is a robust effect on *Kras*-activated tumors treated with miR-34.

$Kras^{LSL\text{-}G12D/+};p53^{flx/flx}$ genetically engineered mice accurately model NSCLC, both in disease progression, and response and

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resistance to conventional therapies.[27–29] As tumor formation in this model depends on two or more signaling pathways that are associated with *let-7* and miR-34, we explored whether combining miR-34 and *let-7* into a single therapeutic could interfere with constitutively active processes in heterogeneous cancer cells to induce greater treatment efficacy. We show that simultaneous supplementation of these two tumor suppressor miRNAs results in an even broader repression of key oncogenes and enhanced efficacy in aggressive NSCLC compared with treatment with the individual miRNAs.

RESULTS

miR-34 and *let-7* synergize in NSCLC cells in culture

To evaluate the combined efficacy of these two master regulators, seven different lung cancer cell lines were transfected with low nanomolar concentrations of *let-7* or miR-34 individually, or half of the dose of each in combination. When transfected with *let-7b* or miR-34a alone, proliferation of cells harboring both *KRAS* and *TP53* open reading frame mutations (in cell lines: H358, H23, and H441) was decreased. Similarly, the combination of half doses of *let-7b* and miR-34a was equally or, in some cases (H441), more effective (Figure 1a and Supplementary Figure 1). Cell lines with only a *KRAS* open reading frame mutation (H460 and A549) or a *TP53* mutation (EKVX) were less affected by the combination. These data suggest that the use of either miRNA alone or in combination is effective in a *KRAS;TP53*-mutated background.

Additional analysis in H441 cells determined that the overall rate of proliferation was decreased more strongly in cells transfected with half the dose of both miRNAs (Figure 1b) relative to cells transfected with *let-7b* or miR-34a individually. To determine whether co-treatment with *let-7b* and miR-34a results in a synergistic effect on cell proliferation, dose-response curves and combination indices were calculated from proliferation data generated from H441 and H23 cells. This analysis confirmed that *let-7b* and miR-34a synergize in H441 cells (Figure 1c) but act additively in H23 cells (data not shown). In H441 cells, combination index scores were well below 1.0 when the effect size was $>15\%$, indicating a synergistic response (combination index H441: at 25% growth inhibition = 0.65, at 20% growth inhibition = 0.74 and at 15% growth inhibition = 0.86). To better characterize the cellular effects of treatment with the miRNA combination in H23 cells, we evaluated cell migration through a matrigel-coated membrane following transfection. H23 cells transfected with either *let-7b* or miR-34a individually were capable of invading equally well or better than cells transfected with a control, scrambled miRNA (Figure 1d). In contrast, H23 cells exposed to the *let-7b*/miR-34a combination showed a reduction in their ability to invade (*P*-values of *let-7b*- and miR-34a-transfected cells relative to: control = 0.06, *let-7b* = 0.07, miR-34a = 0.004).

Tumor suppressor miRNAs often mediate their effects by targeting potent genes in key oncogenic signaling pathways. Therefore, we evaluated the expression of LIN28B, MET and MYC following transfection. MET is a *bona fide* miR-34 target,[12] whereas MYC is reported to be repressed by both miR-34 and *let-7*.[11] *let-7* also represses LIN28.[20,30,31] Specifically in cells transfected with miR-34a, MET levels were reduced, and MYC and LIN28B were downregulated in cells transfected with *let-7b*. These effects were also observed in cells transfected with half the dose of *let-7b* and miR-34a (Figure 1e). Together, the results indicate that combinatorial treatment with *let-7b* and miR-34a results in decreased levels of more key oncogenes than transfecting with either individual miRNA, providing mechanistic evidence consistent with the tumor-suppressive effect of these miRNAs seen above.

Superior effects of the miR-34 and *let-7* combination in NSCLC mouse models *in vivo*

Next, we determined the antitumor activity of the *let-7b*/miR-34a combination *in vivo,* using the $Kras^{LSL\text{-}G12D/+}$;$p53^{flx/flx}$ NSCLC model. This model is the most accurate representation of human NSCLC in disease progression, and response and resistance to conventional therapies, making it an excellent model to use for our studies.[27,28] To induce tumor formation, transgenes in $Kras^{LSL\text{-}G12D/}$;$p53^{flx/flx}$ mice were induced specifically in the lung through intratracheal administration of 10^6 PFU of adenoviral particles expressing Cre recombinase. Following transgene recombination, tumors developed at an accelerated rate with defined nodules present in a subset of animals 5 weeks following Ad-Cre delivery. Both miR-34 and *let-7* levels were confirmed to be low in tumors relative to the expression in normal lung tissue. miR-34b and miR-34c levels were reduced to $<50\%$ of the level found in normal lung tissue (Supplementary Figure 2). *let-7a, let-7b* and miR-34a levels were unchanged at 10 weeks post transgene recombination; however, we did observe a decrease in expression 4 weeks later for *let-7b* in both the lung tissue and in the whole blood (Figures 2f and g; compare baseline to miR-NC). We likely did not observe a decrease in miR-34a owing to the overall low baseline levels of miR-34a in the whole lung (Figure 2d). Interestingly, in the tumor tissue we also observed increased expression of the oncomiRs, miR-19, miR-155 and miR-21 (Supplementary Figure 2). On average, miR-19 was increased five-fold, miR-155 approximately four-fold and miR-21 over two-fold relative to normal lung tissue.

We had previously identified a neutral lipid emulsion (NLE) delivery agent suitable for systemic *in vivo* targeting to the lung (MaxSuppressor, Bioo Scientific and Mirna Therapeutics, Austin, TX, USA) that showed no obvious signs of toxicity.[25,26] Therefore, we tested the efficacy of NLE-encapsulated *let-7b* and miR-34a in the therapeutically resistant $Kras^{LSL\text{-}G12D/+}$;$p53^{LSL\text{-}flx/flx}$ mouse model. We treated animals 10 weeks following transgene recombination, systemically administering 1 mg/kg of NLE-encapsulated *let-7b*, miR-34a or half the dose of each into the tail vein. As a control, the NLE delivery agent alone was also evaluated. Animals were dosed every other day (QOD) for a total of 11 doses, a time when the control animals and some of the miR-34a or *let-7b* individually dosed animals became sick and died. The remaining animals were killed either 24 or 48 h following the final injection and evaluated for lung tumor burden and tumor number (Figure 3). A reduction in tumor nodules was only apparent when animals were administered the combination of 0.5 mg/kg of *let-7b* and 0.5 mg/kg of miR-34a (Figure 3c). In animals treated with the combination, tumor burden decreased from 49 to 31% (Figure 3d) and tumor nodules decreased from an average of 17.6 to 8.25 nodules per left lobe (Figure 3c). Because some animals were too sick to withstand the stress from the injections and died, they were not included in the final histological data set and statistical power was reduced. As such, we conducted a larger and more powerful study to support the use of combinatorial therapy.

The second *in vivo* study used a promising new lipid-based delivery agent, NOV340 (Marina Biotech, Bothell, WA, USA), that accumulates in the lung, liver and spleen of systemically treated mice (AGB, unpublished data and Figures 2d and f). Equally important, the path to the clinic could be quickened as this delivery agent showed limited toxicity in preclinical animal studies[32] and is currently the subject of a phase I clinical trial for MRX34, a NOV340 nanoparticle loaded with miR-34a mimics to treat patients with unresectable primary hepatocellular carcinoma or individuals with unresectable liver metastasis (NCT01829971).[33] Owing to the early demise of some animals in the previous trial we started treatment of animals at 7 weeks post transgene activation instead of at 10 weeks that was followed in the previous trial. This

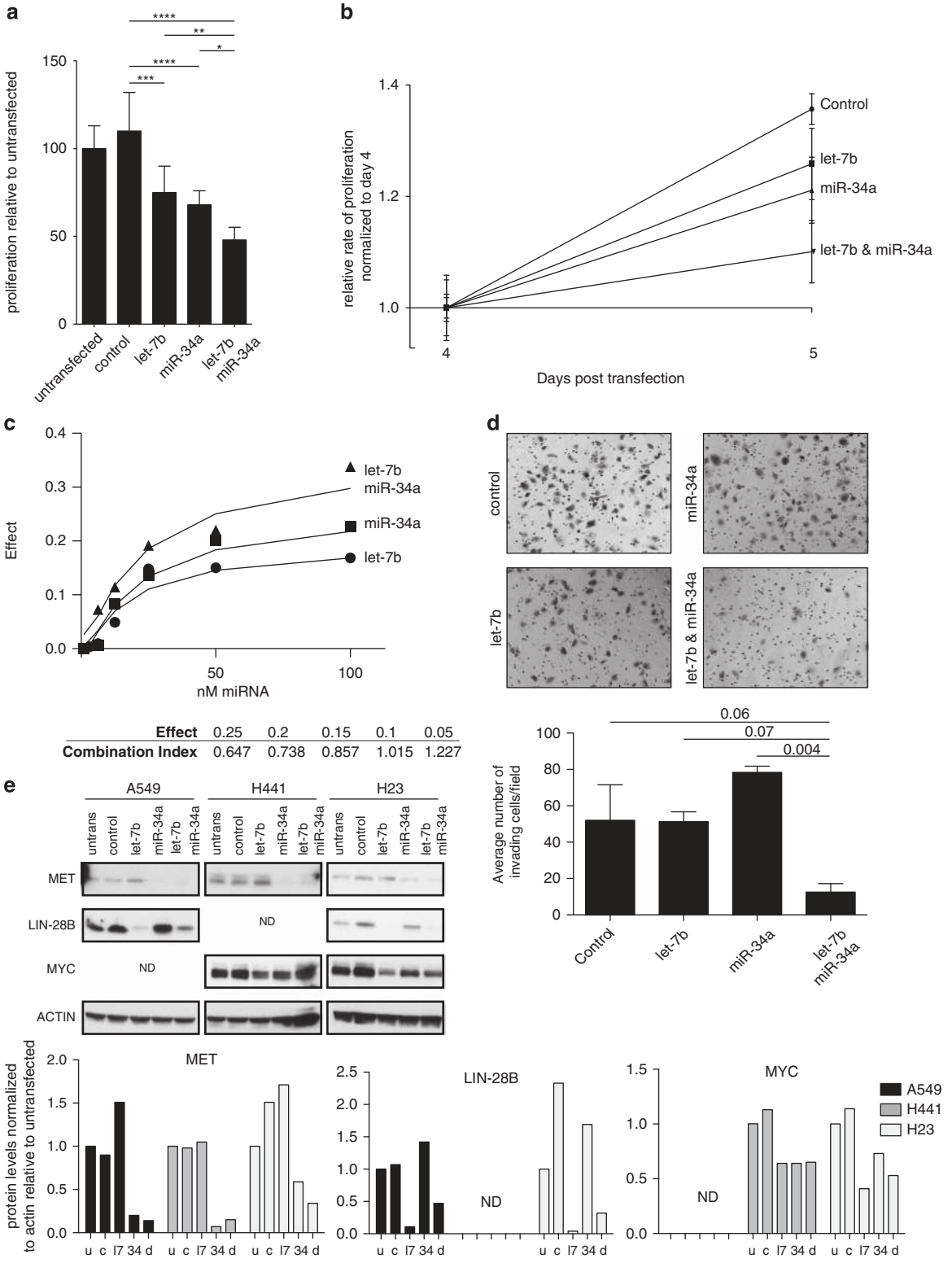

Figure 1. miR-34a and *let-7b* reduce tumor cell proliferation and invasiveness in a synergistic manner. (**a–c**) Cells were transfected with miRNA mimics: control, *let-7b*, miR-34a, or half the dose of each. (**a**) Sulphorhodamine B (SRB) assays were performed 7 days post transfection. Data represent five transfections for each treatment. Error bars depict s.d. (**b**) Change in SRB-stained cells from day 4 to 5 post transfection is shown as a proxy for change in proliferation, $n = 3$ for each treatment. Error = s.d. (**c**) Dose–response curves were created over a wide range of miRNA concentrations (3–100 nM). Effect represents decreased proliferation. (**d**) Invading H23 cells were stained 24 h after seeding. Three fields of view were photographed and the average number of invading cells per field is graphically represented. (**e**) Human lung cancer cell lines, A549, H441 and H23, were transfected with 25 nM of miRNA mimics: control (**c**), *let-7b* (l7), miR-34a (34), or half the dose of both *let-7b* and miR-34a (d) or left untransfected (u). Forty-eight hours following transfection, protein lysates were prepared, resolved, transferred to polyvinylidene difluoride, and evaluated for relevant *let-7b* and mir-34a target genes. Actin serves as a loading control. ND, not detected. Densitometry measurements relative to untransfected cells are shown following normalization to actin. **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001, *****P*-value < 0.0001, unless otherwise indicated, based on one-way analysis of variance with Tukey's *post hoc* correction.

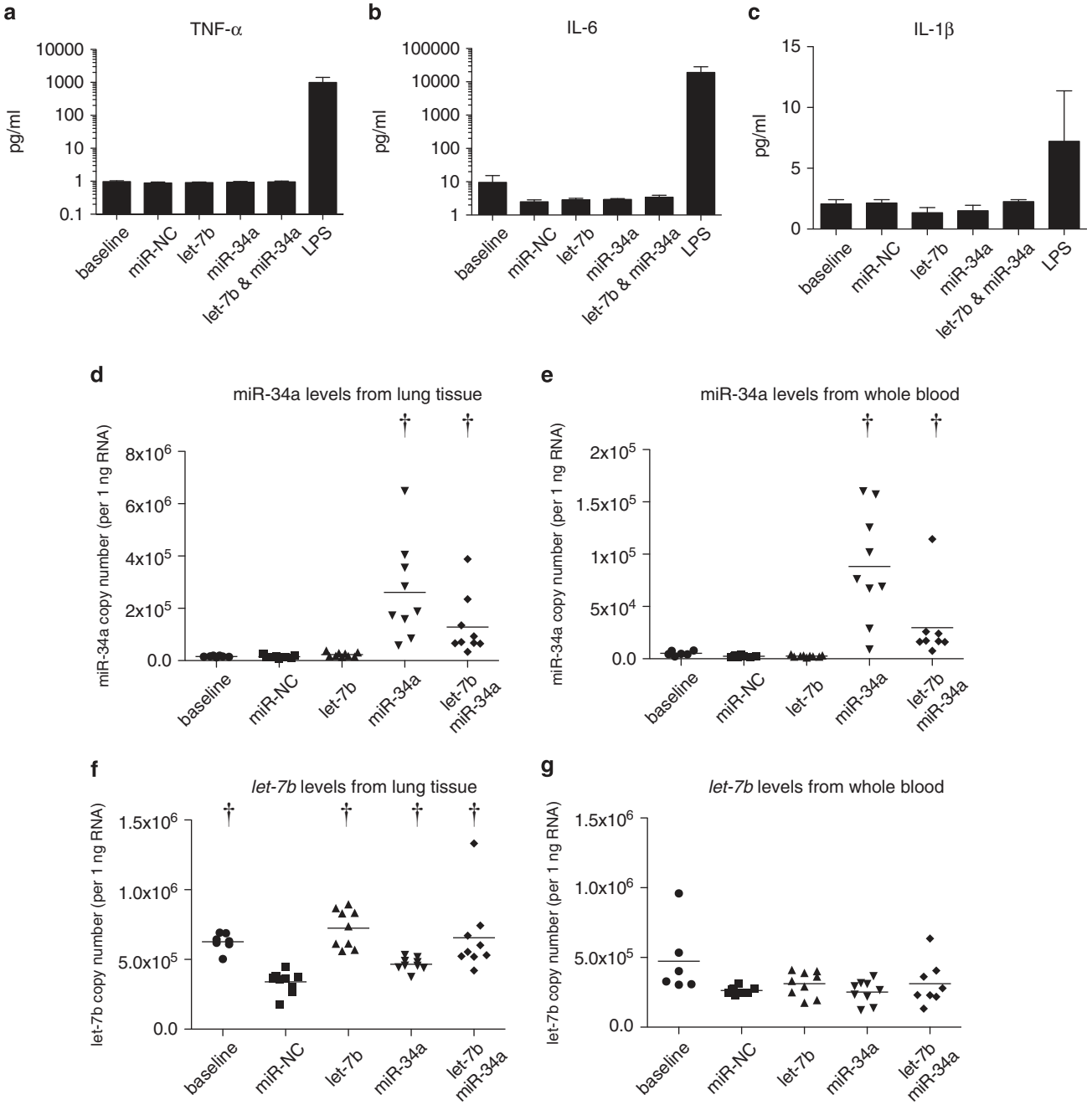

Figure 2. Systemically delivered miRNAs accumulate in whole blood and lung tissue, and do not elevate serum cytokines. Serum, whole blood and lung tissue were harvested 24 or 48 h following the final injection. (**a**–**c**) Serum was evaluated for relevant cytokines: tumor necrosis factor (TNF)α, interleukin (IL)-6 (**b**) and IL-1β (**c**). Serum from animals treated with lipopolysaccharides (LPS) was included as a positive control ($n = 3$). (**d**–**g**) RNA was extracted from lung tissue (**d** and **f**) or whole blood (**e** and **g**) and evaluated for accumulation of miR-34a (**d** and **e**) or and *let-7b* (**f** and **g**) by RT–qPCR. Data are shown as copies per 1 ng of total RNA (baseline: $n = 7$, control: $n = 9$, *let-7b*: $n = 9$, miR-34a: $n = 8$, *let-7b* and miR-34a: $n = 8$). † Statistically significant from miR-NC treated based on one-way analysis of variance of log-transformed data followed by a Dunnett's *post hoc* test (multiple comparison test).

allowed control animals to survive through the completion of the study over the course of 14 total injections QOD (Figure 4a). Similar to the previous studies, animals were administered *let-7b*, miR-34a or half the dose of both miRNA mimics. Formulations were administered intravenously at 1 mg/kg per injection, a dose well below levels that induce toxicities in mice (AGB, unpublished data). Either 24 or 48 h after the final injections, animals were killed and evaluated for tumor burden, signs of toxicity, accumulation of miRNAs in whole blood and lungs, and relevant markers. As expected and in agreement with previous studies, there was no evidence of whole organ toxicity or elevation in any of the serum cytokines: tumor necrosis factor, interleukin (IL)-6 and IL-1b (Figure 2a). Systemic delivery of the individual miRNAs led to marked accumulation of miR-34a and *let-7b* in the lung (Figures 2d and f, P-value < 0.0001 for miR-34a and < 0.0001 for *let-7b*) and miR-34a in the whole blood (Figure 2e, P-value < 0.0001) compared with miR-NC-treated mice. It is of interest to note that *let-7b* levels dropped in both the lung

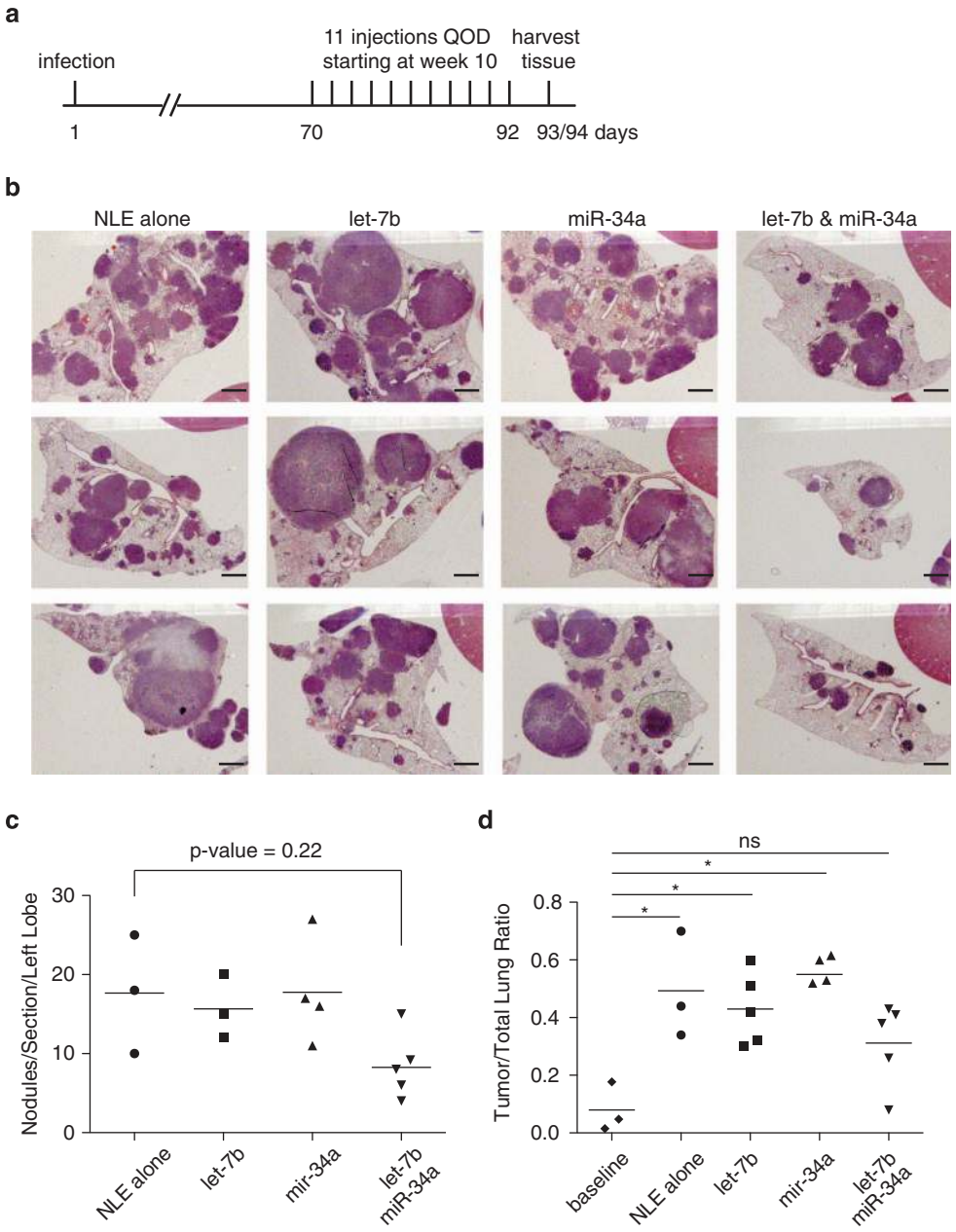

Figure 3. Systemic delivery of miRNAs encapsulated in neutral lipid emulsion slows tumor growth. (**a**) Schematic of dosing schedule. (**b**) Animals were killed 24 or 48 h after the final injection; lungs were perfused, harvested and processed for hematoxylin and eosin staining. Bars, 1 mm (**c**) Tumor nodules were counted from three sections obtained from the left lobe of each animal and are shown as average nodules per section per left lobe. (**d**) Overall tumor burden represents total tumor area averaged from three sections obtained from each treated animal relative to the total area of the lung (baseline: $n = 3$; NLE: $n = 3$; *let-7b*: $n = 5$; miR-34a: $n = 4$; *let-7b* and miR-34a: $n = 5$). (Note: two of the *let-7b* sections were not included in the nodule count as the sections obtained were predominantly one large nodule.) *P-value < 0.05, unless otherwise indicated, based on one-way analysis of variance with Tukey's *post hoc* correction. ns, not significant.

and whole blood as the tumors progressed (Figures 2f and g: baseline animals relative to miR-NC treated animals in lung, P-value = 0.001, and in whole blood, P-value = 0.034). This suggests that *let-7b* expression decreases as the tumors progress and, furthermore, that our systemic administration can restore the levels to at least baseline in the lung. The data for miR-34a also indicate that miRNA levels correlated with miRNA dosing: animals that received a full dose (1 mg/kg) had roughly twice as much miR-34a as animals that were injected with the combination containing half the dose (0.5 mg/kg) of each miRNA. These levels were sustained at least 48 h after the last dose. We compared levels in animals killed 24 or 48 h after the final injection (not shown), which revealed no significant changes between the two time points.

Histological evaluation determined that overall tumor burden was reduced in animals administered the combinatorial therapy (Figures 4b and c and Supplementary Figure 3). The reduced tumor burden was attributed to a decrease in individual tumor size (Figure 4d). The combinatorial administration reduced tumor volume to $< 43\%$ of the control tumors (P-value < 0.0001). Notably, average lung tumor volumes from the dual treated animals were also significantly smaller than average volumes from either of the single miRNA-treated groups (relative to miR-34a: P-value < 0.05, relative to *let-7b*: P-value < 0.01), suggesting that

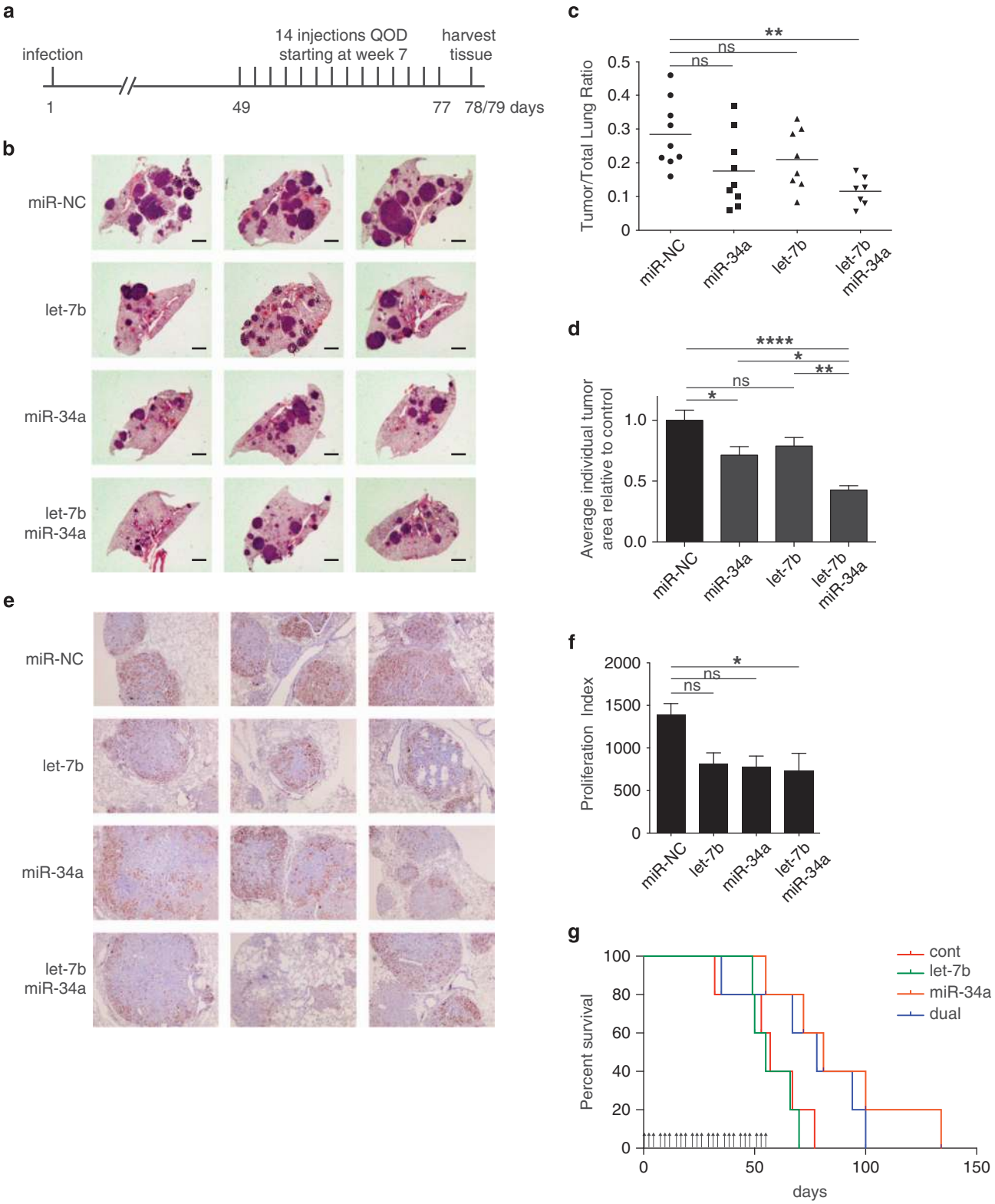

Figure 4. Reduction in tumor size following systemic delivery of combinatorial miRNAs encapsulated in a clinically relevant NOV340 delivery agent. (**a**) Schematic of dosing schedule. (**b**) Representative hematoxylin and and eosin stain of the left lobe of animals from each treatment group (histology from every animal is shown in Supplementary Figure 3). Bars, 1 mm (**c**) Quantification of tumor area relative to total lung area. (**d**) The volumes of individual tumors were recorded and averaged for each treatment group. Each bar represents over 280 tumors from each treatment group. Standard error of the mean is depicted. (**e**) Images of KI-67 staining from representative sections for each treatment group. (**f**) Proliferation index calculated as KI-67 positive cells/mm^2. (c, d, f) *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001 based on one-way analysis of variance with Tukey's *post hoc* correction. ns, not significant. (**g**) Survival data of *Kras*$^{G12D/+}$;*p53*$^{flx/flx}$-treated mice (arrows indicate injection days). Survival P-values = 0.07 for control vs *let-7b* and miR-34 combination and 0.05 for control vs miR-34a following log-rank (Mantel–Cox) test.

combinatorial treatment with *let-7b* and miR-34a has a significant effect on tumor growth. In addition, we observed a decrease of Ki-67 staining in combinatorial-treated tumors, indicating that the tumors exposed to miR-34a and *let-7b* were proliferating at a slower rate (Figure 4).

Individual lung tumors that remained in each of the treatment arms were harvested and used to evaluate the effects of miR-34a and *let-7b* on respective target genes (Supplementary Figure 4). Messenger RNA levels of *Myc*, *Lin28A* and *Lin28B,* three of the genes that were expressed at higher levels in the tumor relative to negative control-treated tissue (miR-NC), were found to be downregulated by exogenous miR-34a and/or *let-7b*. *Bcl2* and *Met*, gene products that did not change during lung tumorigenesis, were also reduced by the combinatorial treatment and miR-34a and/or *let-7b*. The results suggest that the delivery of miRNA mimics was sufficient to repress their downstream targets. Importantly, the collective response on target genes was sustained in the group treated with the combination, showing that through combinatorial miRNA administration we were able to downregulate more target genes than through the use of each miRNA alone. The relative decreases in the transcript levels *in vivo* were similar to the reduced protein levels from the human cell culture experiments (Figure 1e).

miR-34 and *let-7* improve survival in *Kras; p53* mutant mice

Although a few lung agents with Food and Drug Administration approval lead to modestly increased survival in this mouse model,[27] no new clinically relevant agents have shown a similar benefit.[29] Therefore, because this mouse model is the most accurate representation of human response to Food and Drug Administration approved therapeutics for NSCLC we sought to evaluate whether the new combinatorial miRNA therapy could increase the average survival of *Kras;p53* mice. Transgenes were activated in the lungs of the mice as before and tumors were allowed to develop for 6 weeks before animals were separated into four treatment arms. Each animal received 10 mg/kg NOV340 per intravenous miRNA injection, such that miRNAs administered alone were given at 10 mg/kg, and each miRNA in the combination was used at 5 mg/kg. Animals were dosed three times per week for a total of 8 weeks. At this time point, ~ 50% of the control animals had died. Kaplan–Meier survival curves, shown in Figure 4g, demonstrate that there was a clear survival advantage (~40% increase) for the animals administered the combination of half miR-34 and half *let-7b* (P-value = 0.07), and for the miR-34a alone group (P-value = 0.05). Animals administered the control miRNA had a median survival of 57 days following the first injection and miR-34a treatment alone increased the median survival to 81 days. Whereas *let-7b* treatment alone had no effect on increasing survival (median of 55 days), animals administered the half-dose combinatorial therapy had a median survival of 78 days. Together, these results indicate that treatment with miR-34a or the half-dose combination of *let-7b* and miR-34a can increase average survival in this model of aggressive NSCLC.

DISCUSSION

Lung cancer is the leading cause of cancerous deaths worldwide with non-small cell lung cancer making up the bulk of newly identified cases. Unfortunately, current therapeutics fail to treat the majority of individuals with this disease, even when using recently developed targeted therapeutics. Primary or secondary resistance from targeted agents can be attributed to acquired mutations in the target that render the initial therapeutic less effective or ineffective and/or compensatory mutations in other pathways that allow the cancer cells to overcome the effects of the targeted therapy. Combinatorial microRNA therapeutics is a new frontier in gene therapy that has the ability to overcome these challenges by targeting multiple components of key oncogenic pathways.

The study we present here is the first to show that a combination of two biologically relevant, tumor-suppressive miRNAs is superior in its ability to repress oncogene expression, preventing proliferation and invasion of cancer cells in culture, to inhibit tumor proliferation *in vivo* and to confer a survival advantage. Half the dose of each of these miRNAs was capable of repressing relevant biological targets in cells and *in vivo,* yet exhibited undetectable toxicity. We have performed these timely *in vivo* studies using a miRNA delivery agent already in clinical trials, which should accelerate the translation of this combinatorial miRNA therapeutic approach into the clinic. Although it still needs to be explored, it is expected that the tumors exposed to the combinatorial therapy could now be more sensitive to conventional therapies based on the coordinated downregulation of both *let-7* and miR-34 target genes involved in chemoresistance, relative to the individually dosed animals. Furthermore, it is expected that this miRNA combination, which hits multiple relevant biological targets, would prevent the onset of acquired resistance that has been observed with other therapies tested in this model.[29]

Although the study presented here shows a similar effect on survival between animals exposed to the full dose of miR-34a or those treated with half the dose of miR-34a and *let-7b*, this effect may be attributed to the low levels of miR-34a in the baseline treated animals. When the miRNAs are supplemented, the change in miR-34a levels is much larger than that of *let-7b*, which does not change strongly (Figures 2d–g). It is possible that in this experimental system the net effect of the large change in miR-34a levels is driving the survival effect. It is however likely that the combinatorial treatment would display increased efficacy in humans by impairing the development of secondary resistance. The accumulation of mutations in human tumors may render the tumor resistant to a single agent. By simultaneously targeting multiple key factors in the oncogenic response, the combinatorial approach presented here will likely diminish the occurrence of acquired resistance. Collectively, this is a first step in the progression toward the adoption of a combinatorial miRNA approach as a less toxic and more direct method to target multiple biologically relevant pathways that tumor cells have become addicted to.

METHODS

Cell culture and transfection

All cell lines were obtained from American Type Culture Collection and were cultured in Roswell Park Memorial Institute media (Invitrogen, Carlsbad, CA, USA) supplemented with fetal bovine serum and penicillin/streptomycin, following standard tissue culture procedures. Cells were maintained at 37 °C in a humidified 5% CO_2 environment and passaged every 3–4 days. Cells were transfected with miRNA mimics (Ambion, Life Technologies, Grand Island, NY, USA) using DharmaFECT 1 (Thermo Scientific, Waltham, MA, USA). For sulforhodamine B experiments, 1000 cells were seeded per well in 96-well cell culture dishes. The following day either 25 or 50 nM final concentration of miRNA mimics in various combinations was complexed with 0.5 µl of DharmaFECT 1 and added to the cells in a final volume of 100 µl of serum- and antibiotic-free media. Transfection media was replaced with complete growth media 4 h later. In all cases, control miRNA was supplemented to keep the entire miRNA level consistent between transfection conditions. For protein lysate retrieval, cells were seeded in 24-well plates and transfected as above except that the DharmaFECT 1 concentration was increased to 2 µl/well.

Sulforhodamine B assays

Cells cultured in 96-well plates were fixed with 10% tricholoroacetic acid in complete media and stained for 30 min with 0.4% (wt/vol) sulforhodamine B dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid and protein-bound dye was extracted with 10 mM

unbuffered Tris base for determination of absorbance at 490 nm using a spectrophotometer.

Trans-well migration assays

Transfected H23 cells plated at 1×10^5 in 100 µl serum-free medium were assayed for migration using BD BioCoat Invasion Chambers (BD Biosciences, San Jose, CA, USA) with 8 µm pores, according to the manufacturer's instructions. Serum-containing media was added to the outside of the chamber to act as a chemoattractant. Briefly, cells were incubated for 24 h to allow for migration across the pores, washed with phosphate-buffered saline, treated with 3.7% formaldehyde in phosphate-buffered saline, washed again and then treated with methanol. After another wash, a 1:20 dilution of Giemsa stain was used to stain the cells, before the cells were washed a final time. Migrating cells were imaged at 10× magnification. For quantification, three fields of view for each transfection were counted and averaged. Three biological replicates were performed.

Protein extraction and immunoblotting

Forty-eight hours following transfection, cells were lysed with radio-immunoprecipitation assay buffer (1x phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate), supernatant was collected, 50 µg of protein was resolved on 12% TGX gels (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes. Primary antibodies for detection included Lin28B (1:500, Cell Signaling, Danvers, MA, USA, 4196), Met (1:500, Cell Signaling, 3127) and Myc-Xp (1:500, Cell Signaling, 5605). Following incubation with secondary antibodies, membranes were washed and signal was acquired using West Dura (Promega, Madison, WI, USA) and exposure of membranes to film. Intensities were quantified using ImageJ Software (NIH).[34]

Kras;p53 animals, induction of tumor formation and treatment

Both $Kras^{LSL\text{-}G12D/+}$ (strain 01XJ6) and $Trp53^{flx/flx}$ (strain 01XC2) mice were obtained from the National Cancer Institute of Frederick Mouse Repository. The *Kras* allele was crossed into the *Trp53* background, FVB.129. Newly acquired double heterozygotes were backcrossed to the *Trp53* strain for four generations before homozygosing 3 the $Trp53^{flx}$ allele. Transgenes in double mutant animals were induced based on the method of DuPage *et al*.[28] Briefly, animals were anesthetized with a mixture of ketamine and xylazine and intratracheally intubated. Adenoviral particles (10^6 PFU) encoding for Cre recombinase were precipitated with $CaCl_2$ and 65 µl of precipitated virus was delivered directly to the lungs through the catheter.

In all cases miRNA mimics were delivered systemically through the tail vein. The NLE was prepared as previously described.[26] Mimics encapsulated in NOV340 were supplied by Mirna Therapeutics. For tumor burden studies, following transgene recombination, animals were administered either 1 mg/kg of NLE-encapsulated miRNA mimics every other day for a total of 11 injections or 1 mg/kg of mimics encapsulated in NOV340 every other day for a total of 14 injections. Twenty-four or forty-eight hours after the final injection animals were killed. Whole blood and serum were obtained and lungs were perfused with saline, harvested, fixed in 10% buffered formalin and paraffin-embedded according to standard procedures. Sections were stained by hematoxylin and eosin and evaluated using a Zeiss dissection microscope (Zeiss, Jena, Germany), AxioCam MRc 5 camera (Zeiss) and AxioVision 4.7.1 imaging software (Zeiss). Tumor burden represents the tumor area relative to the total lung area obtained from three independent sections for each animal. For survival studies, 10 mg/kg of miRNA mimics (5 mg/kg of *let-7b* and miR-34a for the combination) were delivered systemically through the tail vein three times per week for 8 weeks. Animal survival was monitored throughout the remainder of the study. Log-rank tests were performed to determine significance. All animals were housed in the Yale University Animal Facility under the guidelines held by the Institutional Animal Care and Use Committee.

Ki67 IHC

Tissue sections (3 µm) were adhered to PLUS slides and deparaffinized at 70 °C for 30 min followed by standard xylene and alcohol clearing steps. Slides were immersed in 90 °C Target Retrieval Solution (Dako, Glostrup, Denmark, Europe) for 20 min, followed by a gradual cooling step by placing the hot solution at room temperature for 10 min. Slides were then rinsed with wash buffer (Dako). The next steps were followed sequentially by a rinse with wash buffer: block slides for endogenous peroxidase activity using dual enzyme block (Dako) for 5 min, Fc receptor block (Innovex, CA, USA) for 1 h, background buster (Innovex) for 30 min, Ki67 primary antibody (Abcam, Cambridge, MA, USA, cat# ab8191) at 1:500 dilution for 1 h , secondary reagent (Rabbit Polymer, Dako) for 1 h , envision +horseradish peroxidase (Dako) for 30 min, 3,3'-diaminobenzidine substrate for 10 min and automated hematoxylin (Dako) counter stain for 6 min. Slides were dehydrated by rinsing them once in 70% alcohol, twice in 95% alcohol and three times in 100% alcohol. Slides were dipped three times in xylene and coverslipped using Richard–Allen Scientific Mounting Medium (Thermo Scientific, MA, USA). KI-67 positive cells, in 2 mm sections, were counted and averaged across all animals in treatment arm.

RNA isolation and qRT–PCR

Total RNA from whole blood and tissues was isolated using the mirVana PARIS RNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Briefly, 250 µl of blood lysate (250 µl whole blood+250 µl denaturing solution) or 600 µl of tissue homogenate (1 ml denaturing solution added to slightly thawed tissue sample and homogenized) was combined with an equal volume of acid–phenol, vortexed and centrifuged at 13.2k *g* for 15 min. One half of the resultant aqueous phase was transferred to a fresh tube and the remaining protocol was followed according to the manufacturer's instructions. A volume of 50 µl of heated (95 °C), nuclease-free water was used to elute the RNA from the column.

For the detection and quantification of miRNA from whole lungs and blood by quantitative reverse trascription–PCR, isolated RNA was converted to complementary DNA (cDNA) using MMLV-RT (Invitrogen) under the following conditions: 4 °C for 15 min; 16 °C for 30 min; 42 °C for 30 min; and 85 °C for 5 min. TaqMan microRNA assays were used for expression analysis of hsa-miR-34a and hsa-*let-7b* (Life Technologies, Carlsbad, CA, USA). Following cDNA synthesis, qPCR was performed on 2 µl of cDNA on the ABI Prism 7900HT SDS (Life Technologies) using Platinum Taq Polymerase (Invitrogen) under the following cycling conditions: 95 °C for 1 min (initial denature); then 50 cycles at 95 °C for 5 s; and 60 °C for 30 s. Additions to the manufacturers' reagents include dimethyl sulfoxide (final concentration of 6%) and tetramethylammoniumchloride (final concentration of 50 mM in both RT and PCR) to improve the slope, linearity and sensitivity of the assays.

Absolute quantitation was determined as copy number per nanogram of RNA input by extrapolation to the miR-34a and *let-7b* standard curves. Final values were calculated by factoring in losses during the acid–phenol extraction (carryover of only half of the aqueous phase), elution volume, volume of RNA eluent into the reverse transcription reaction and starting mass of RNA.

For the detection and quantification of miRNAs and messenger RNA targets by qRT–PCR from individual lung tumors, isolated RNA was converted to cDNA using miScript II RT Kit (Qiagen, Venlo, Netherlands). Following cDNA synthesis, qPCR was performed on 2 µl of cDNA on the Roche 480 using Qiagen's miScript SYBR Green Assay Kit. Qiagen probes were used for expression analysis of all genes of interest. Relative quantification of miR-34a and *let-7b* and their targets was determined as the concentration (fold-change) of the genes of interest compared with the normal lung tissue. Rnu6B (miRNA), and Gapdh and Actin (messenger RNA) were used as housekeeping genes to normalize for RNA input variances into the reverse transcription reaction.

Serum cytokines

Mice serum samples were used to test for IL-6, IL-1b and tumor necrosis factor concentrations using the cytokine assay kit from R&D systems according to manufacturer's instructions (Fluorokine MAP Mouse Base Kit Cat# LUM000, mouse IL-1b Cat# LUM401, mouse IL-6 Cat# LUM406, mouse tumor necrosis factor-αβ Cat# LUM410). Briefly, serum samples were thawed and cleared from debris by centrifugation at 1000 *g* for 10 min before the analysis. All samples or standards were added to a 96-well plate together with assay buffer and cytokine beads. Plates were gently shaked for 3 h at room temperature and spun as above. After removal of the supernatant, wells were washed. A detection antibody was added and the plates were incubated for 1 h at room temperature with shaking. Plates were rinsed and incubated with streptavidin–phycoerythrin for 30 min at room temperature. The wells were washed and the captured beads were re-suspended to acquire data using the Luminex 100 IS2.2 detection

system (Austin, TX, USA). Cytokine standard curves were used to calculate the cytokine concentrations in serum samples (pg/ml).

CONFLICT OF INTEREST

KK, JZ and AGB are employees of Mirna Therapeutics, which develops miRNA-based therapies. SD is a former employee of Mirna Therapeutics. FJS is a consultant for Mirna Therapeutics. KK, JZ, AGB and FJS are shareholders of Mirna Therapeutics. The remaining authors declare no conflict of interest.

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REFERENCES

1 National Lung Screening Trial Research Team, Aberle DR, Berg CD, Black WC, Church TR, Fagerstrom RM *et al.* The National Lung Screening Trial: overview and study design. *Radiology* 2011; **258**: 243–253.
2 Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M *et al.* Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006; **9**: 189–198.
3 Lin P-Y, Yu S-L, Yang P-C. MicroRNA in lung cancer. *Br J Cancer* 2010; **103**: 1144–1148.
4 Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A *et al.* RAS is regulated by the let-7 microRNA family. *Cell* 2005; **120**: 635–647.
5 Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H *et al.* Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res* 2004; **64**: 3753–3756.
6 Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE *et al.* p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 2007; **17**: 1298–1307.
7 Lodygin D, Lodygin D, Tarasov V, Tarasov V, Epanchintsev A, Epanchintsev A *et al.* Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle* 2008; **7**: 2591–2600.
8 Gallardo E, Navarro A, Viñolas N, Marrades RM, Diaz T, Gel B *et al.* miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer. *Carcinogenesis* 2009; **30**: 1903–1909.
9 Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* 2007; **21**: 1025–1030.
10 Sampson VB, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P *et al.* MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res* 2007; **67**: 9762–9770.
11 Wei JS, Song YK, Durinck S, Chen Q-R, Cheuk ATC, Tsang P *et al.* The MYCN oncogene is a direct target of miR-34a. *Oncogene* 2008; **27**: 5204–5213.
12 He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y *et al.* A microRNA component of the p53 tumour suppressor network. *Nature* 2007; **447**: 1130–1134.
13 Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A *et al.* Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle* 2007; **6**: 1586–1593.
14 Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N *et al.* Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 2007; **26**: 731–743.
15 Chang T-C, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH *et al.* Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 2007; **26**: 745–752.
16 Zhao J, Lammers P, Torrance CJ, Bader AG. TP53-independent function of miR-34a via HDAC1 and p21(CIP1/WAF1.). *Mol Ther* 2013; **21**: 1678–1686.
17 Kasinski AL, Slack FJ. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer* 2011; **11**: 849–864.
18 Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol* 1999; **216**: 671–680.
19 Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004; **15**: 185–197.
20 Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. *Science* 2008; **320**: 97–100.
21 Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L *et al.* The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 2008; **7**: 759–764.
22 Trang P, Medina PP, Wiggins JF, Ruffino L, Kelnar K, Omotola M *et al.* Regression of murine lung tumors by the let-7 microRNA. *Oncogene* 2009; **29**: 1580–1587.
23 Kumar MS, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA *et al.* Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA* 2008; **105**: 3903–3908.
24 Kasinski AL, Slack FJ. miRNA-34 prevents cancer initiation and progression in a therapeutically resistant K-ras and p53-induced mouse model of lung adenocarcinoma. *Cancer Res* 2012; **72**: 5576–5587.
25 Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown D *et al.* Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34. *Cancer Res* 2010; **70**: 5923–5930.
26 Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D *et al.* Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther* 2011; **19**: 1116–1122.
27 Singh M, Lima A, Molina R, Hamilton P, Clermont AC, Devasthali V *et al.* Assessing therapeutic responses in Kras mutant cancers using genetically engineered mouse models. *Nat Biotechnol* 2010; **28**: 585–593.
28 DuPage M, Dooley AL, Jacks T. Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase. *Nature Protoc* 2009; **4**: 1064–1072.
29 Xue W, Meylan E, Oliver TG, Feldser DM, Winslow MM, Bronson R *et al.* Response and resistance to NF-κB inhibitors in mouse models of lung adenocarcinoma. *Cancer Discov* 2011; **1**: 236–247.
30 Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE *et al.* The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature* 2000; **403**: 901–906.
31 Newman MA, Thomson JM, Hammond SM. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* 2008; **14**: 1539–1549.
32 Bader AG. miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet* 2012; **3**: 120.
33 Bouchie A. First microRNA mimic enters clinic. *Nat Biotechnol* 2013; **31**: 577.
34 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; **9**: 671–675.

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