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Identification of cell cycle-targeting microRNAs through genome-wide screens

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ABSTRACT

By performing nine genome-wide microRNA (miRNA) screens, we recently uncovered a new class of miRNAs, which target multiple cyclins and cyclin-dependent kinases (CDKs). Systemic delivery of selected cell cycle-targeting miRNAs to mouse xenograft models resulted in potent anti-tumorigenic effects without affecting animals' health. Here, we provide an in-depth description of our miRNA screening methodology, analyses of selected cell cycle-targeting miRNAs, and discuss why miRNA therapy might be a viable therapeutic option for cancer patients.

Introduction

Cyclin-CDK complexes phosphorylate a number of proteins necessary for progression of cells through the cell cycle. In the early G1 phase, mitogenic stimulation elevates the levels of D-type cyclins (cyclins D1, D2 and D3), which bind and activate the cyclin-dependent kinases CDK4 or CDK6. Later during the cell cycle, E-type cyclins (cyclins E1 and E2) activate CDK2 and contribute to entry of cells into the DNA synthesis (S phase). Progression through the S phase is driven by cyclin A2-CDK2 kinase, while cyclin B-CDK1 regulates multiple mitotic events preceding cell division.[1,2]

Cyclins and CDKs are frequently overexpressed in human cancers.[1,3-7] Analyses of several murine cancer models revealed the requirement for specific cyclins and CDKs both in tumor initiation as well as in tumor maintenance.[8-17] This line of research has recently been translated from bench-to-bedside through the use of CDK inhibitors in cancer treatment. Two inhibitors of CDK4 and CDK6, palbociclib and ribociclib have received approval from US Food and Drug Administration (FDA) for treatment of hormone receptor-positive advanced or metastatic breast cancers. The FDA approval was preceded by clinical trials in which cancer patients receiving CDK4/6 inhibitors experienced prolonged progression-free survival.[18-22] The third CDK4/6 inhibitor, abemaciclib, has completed a clinical phase 3 trial for hormone receptor-positive advanced breast cancer, also resulting in significantly improved progression-free survival of patients.[23] However, when reported, there was little effect on overall survival, possibly due to compensatory actions by other cyclins and CDKs in the face of CDK4/6 inhibition.[19] Hence, the use of agents targeting multiple cell cycle factors may offer improved therapeutic efficacy by delaying, possibly preventing, the emergence of resistant tumor cell populations.

MicroRNAs (miRNAs), through their property to repress several different transcripts, might represent suitable tools to simultaneously target multiple components of the core cell cycle machinery.[24] However, the therapeutic application of miRNAs in combating diseases, including cancer, has been limited so far. The major obstacles are related to difficulties with efficient in vivo delivery and the wide targeting spectrum of many miRNAs.[25-27] Here, we present a follow-up of our recent study describing identification of a novel class of cell cycle-targeting miRNAs, and their successful therapeutic application in mouse xenograft models.[28]

Results

Genome-wide screens for miRNAs regulating 3′UTRs of cyclins and CDKs

To identify miRNAs directly regulating the core cell cycle machinery, we developed a luciferase-based screening methodology in which we cloned the longest annotated 3′UTRs of cyclins D1, D2, D3, E1, E2, CDK1, CDK2, CDK4 and CDK6 into a dual firefly/renilla luciferase reporter vector. Cloned 3′UTRs were subsequently stably expressed in U2OS cells, thereby generating nine different 3′UTR reporter cell lines.[28] Each reporter cell line expressed the firefly luciferase gene placed upstream of the respective 3′UTR. In addition, cells expressed the renilla luciferase driven by the SV40 promoter. In these reporter cell lines, repression of the 3′UTR by a particular miRNA is expected to reduce the firefly luciferase expression, thereby decreasing the firefly/renilla luciferase ratio.

In order to validate each reporter cell line, we turned to miRNAs previously reported to target a particular cyclin or CDK. For example, numerous miRNAs have been described to repress cyclin D1 expression, among which miR-15a is particularly well documented.[29-35] miR-15a targets two conserved sites in the distal end of the cyclin D1 3′UTR (nucleotides 1961–1967 and 2033–2040 in cyclin D1 3′UTR).[36] Premature cleavage and polyadenylation of 3′UTRs are frequently observed in tumors and exploited by cancer cells to avoid miRNA regulation.[37,38] Indeed, the cyclin D1 transcript has been reported to harbor premature cleavage and polyadenylation sites in tumor cells that exclude miR-15a-binding sites.[38] We used miR-15a to evaluate the response of our cyclin D1-3′UTR reporter cell line to miRNA expression. Ectopic expression of miR-15a resulted in a robust (>2-fold) repression of normalized firefly luciferase expression (i.e., firefly/renilla ratio) compared to a scrambled negative miRNA control, with satisfying Z scores (data not shown).

We next proceeded with screening the entire library of 885 mimic miRNAs, representing essentially all annotated miRNAs in the human genome at the time our screens were performed (miRBase v.14), against cyclin D1-3′UTR cells. The screen was carried out in triplicate, with each replicate on a separate plate, on a total of sixty 96-well plates. miR-15a consistently scored as one of the top hits regardless of plate identity (Fig. 1A and B, red dots), confirming the technical robustness of the screen. This was further visualized by pairwise correlations of the three replicates from the cyclin D1 screen (Fig. 1C-E). Using a criterion of at least 40% repression, we found that nearly 100 miRNAs regulated the cyclin D1 3′UTR.[28] Similar screens were performed for miRNAs regulating other major cyclins and CDKs (a total of nine screens).[28]

Identification and analyses of cell cycle-targeting miRNAs

By analyzing the results of the nine 3′UTR screens, we uncovered a novel, previously unanticipated class of miRNAs targeting all or nearly all major cyclins/CDKs and named this group "cell cycle-targeting miRNAs".[28] We further demonstrated that cell cycle-targeting miRNAs displayed reduced expression in several cancer types as compared to the adjacent normal tissue.[28] To test for a possible connection between cell cycle-targeting miRNAs and the levels of cyclins/CDKs in vivo, we correlated the expression of each miRNA present in our
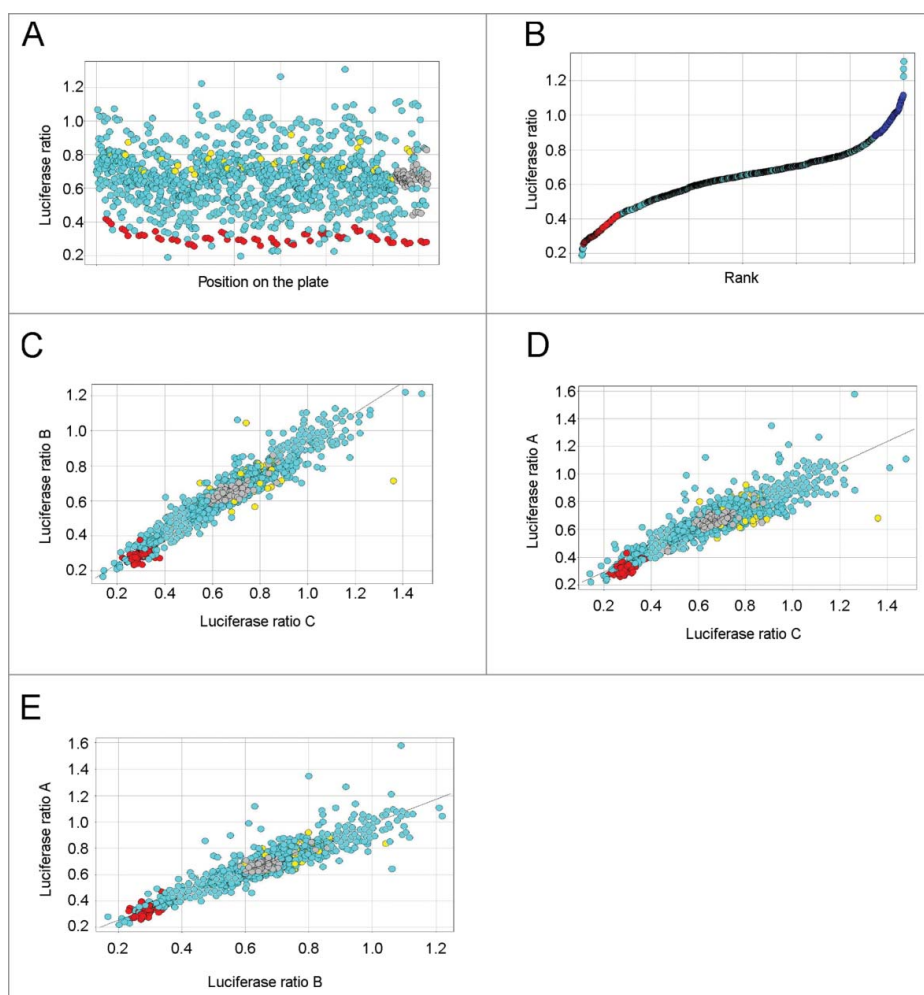

Figure 1. Screening for miRNAs regulating cyclin D1 3′UTR. (A) A scatter plot displaying average firefly to renilla luciferase ratios across all screening plates. Red dots represent a positive control, miR-15a. Yellow dots represent a negative control. Grey dots represent wells without miRNAs. (B) Rank plot displaying average firefly to renilla luciferase ratios for all miRNAs present in the library. Red dots represent a positive control, miR-15a. (C-E) Correlation plots of firefly to renilla luciferase ratios between replicates A, B and C of the cyclin D1 3′UTR screen. A simplified version of panel E was previously shown in Figure S1A in Hydbring et al.[28]
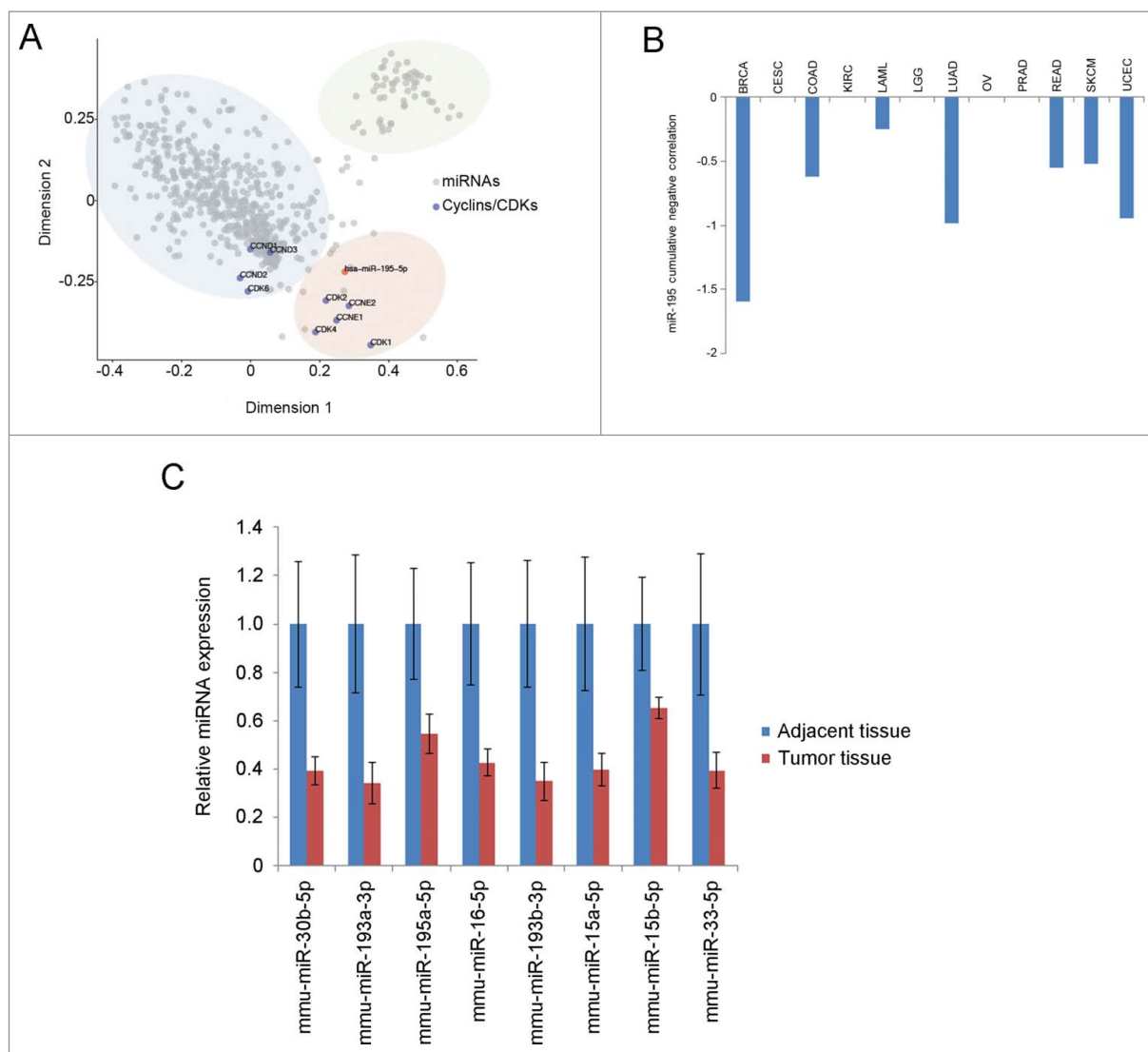
Figure 2. Analysis of cell cycle-targeting miRNAs. (A) Multi-dimensional scaling (MDS) analysis to visualize anti-correlations between expression levels of cell cycle-targeting miRNAs and cyclin/CDK mRNAs (expression data was from TCGA). Grey dots represent all miRNAs, blue dots depict the indicated cyclins and CDKs. The distance between a miRNA dot and a dot representing a cyclin or a CDK indicates anti-correlation in expression (shorter distance – stronger anti-correlation) for this miRNA and a given cyclin or CDK. The three clouds are highlighted by hand to visualize clusters naturally arising from the MDS dimension reduction. The plot illustrates that expression of some miRNAs strongly anti-correlates with multiple cyclins/CDKs. miR-195-5p is highlighted in orange. (B) Cumulative anti-correlations between miR-195-5p expression levels and transcript levels of the nine cyclin/CDKs. BRCA, breast carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; COAD, colon adenocarcinoma; KIRC, kidney renal carcinoma; LAML, acute myeloid leukemia; LGG, lower grade glioma; LUAD, lung adenocarcinoma; OV, ovarian carcinoma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SKCM, skin cutaneous melanoma; UCEC, uterine corpus endometrial carcinoma. (C) miRNA expression analysis of eight cell cycle-targeting miRNA in mouse liver tumor nodules (N = 14) and adjacent tissue (N = 6). Mouse hepatocellular carcinoma was induced by hydrodynamic tail vein injection of DNAs encoding c-Met and constitutive active β-catenin.

screening library (885 miRNAs) with transcript levels of nine cyclins/CDKs in 12 cancer types annotated in the Cancer Genome Atlas (TCGA). Multi-dimensional scaling analysis revealed three distinct clusters of miRNAs in relation to cyclin/CDK mRNAs, with shorter distance between a given miRNA and a given cyclin/CDK representing stronger anti-correlation between their expression levels (Fig. 2A). Interestingly, miR-195-5p, a cell cycle-targeting miRNA identified in our screens, displayed the strongest average anti-correlation with the levels of cyclins/CDKs (Fig. 2A, the brown cluster). A more detailed analysis revealed that of all cancer types analyzed, breast carcinomas displayed the strongest cumulative anti-correlation between miR-195-5p and cyclin/CDK transcript levels (Fig. 2B).

We further investigated the expression of a panel of cell cycle-targeting miRNAs in a genetic mouse model of hepatocellular carcinoma, driven by constitutively active β-catenin (a transcriptional activator of cyclin D1[39,40]) and a receptor tyrosine kinase c-Met. In this model, aggressive liver cancer is induced within 6 weeks following hydrodynamic tail vein injection of DNA encoding constitutively active β-catenin and c-Met.[41] Mice were euthanized 10 weeks after hydrodynamic tail vein injections, and liver tumor nodules along with surrounding liver tissue were analyzed for expression of eight selected cell cycle-targeting miRNAs. We observed reduced expression of all eight miRNAs in tumor nodules compared to non-tumor tissue (Fig. 2C). Five of these miRNAs displayed reduced expression in multiple human cancer types,[28] indicating that

repression of cell cycle-targeting miRNAs may represent a general molecular mechanism operating in cancer cells. These results further suggest that a broad dampening of cell cycle-targeting miRNA expression may contribute to initiation of tumorigenesis.

Prediction of tumors' response to cell cycle-targeting miRNAs

We profiled the response of 122 cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) to four cell cycle-targeting

miRNAs, miR-193a-3p, miR-195-5p, miR-214-5p and miR-890.[28] By intersecting our observed response of these cell lines to cell cycle-targeting miRNAs (i.e., reduction in cell number compared to a scrambled miRNA control) with publicly available mRNA expression data of these cell lines (from CCLE), we derived an elastic net regression model to predict the response of tumors to cell cycle-targeting miRNAs. In order to create this algorithm, 90 cell lines (out of 117 analyzed) were randomly selected as training data to learn the relationship between their gene expression profiles and their response to cell cycle-targeting miRNAs. The expression of individual genes was first correlated
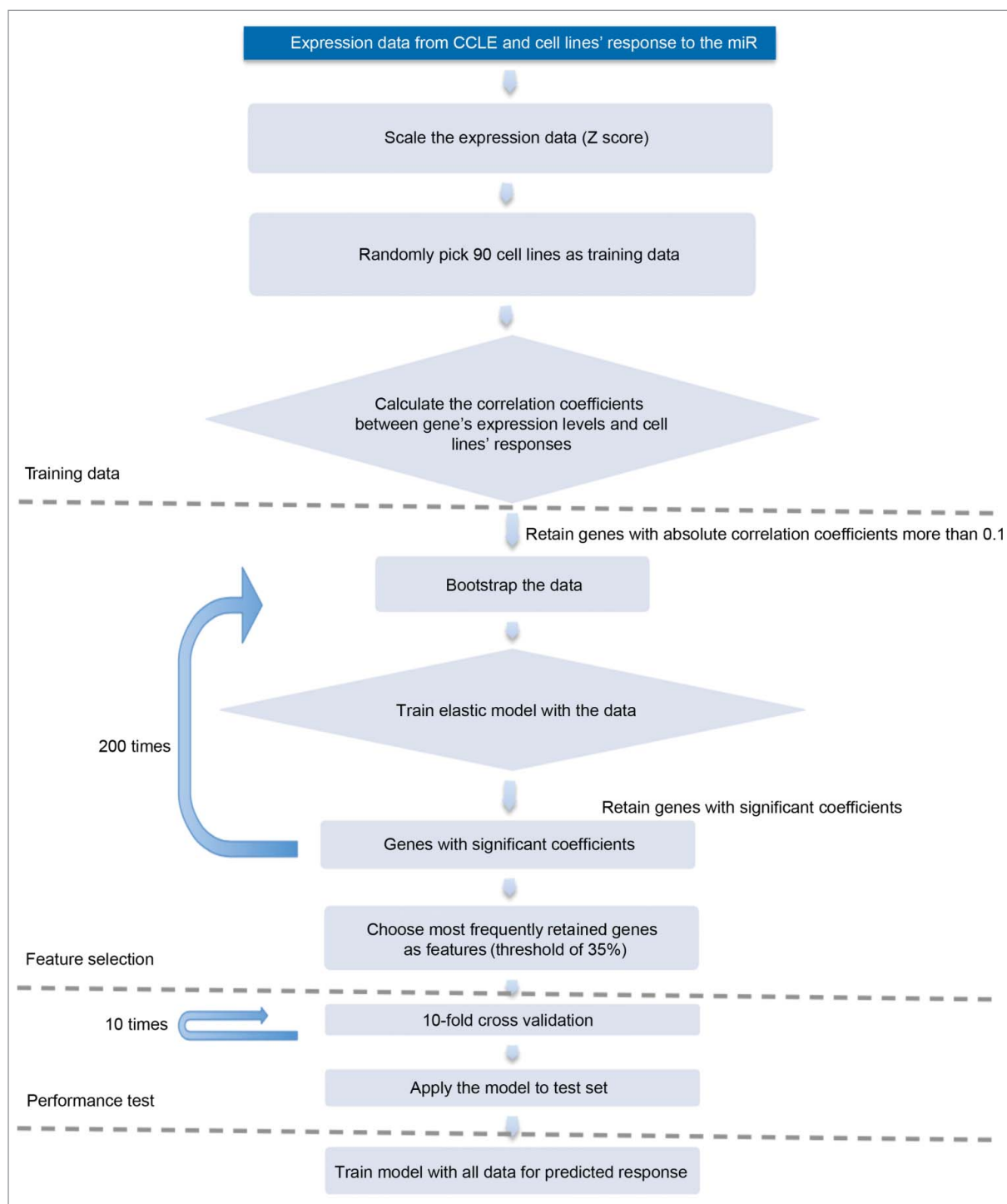

Figure 3. The work-flow for development of the response prediction algorithm.

to cell lines' response to miRNAs, and genes with correlation coefficient >0.1 were kept to train the elastic net regression model (Fig. 3). We ran 200 times bootstrap and applied elastic net model to the resampled data. Each time only a small fraction of genes had significant coefficients and were retained in the model. We then counted the number of times a gene was retained and choose the most frequently (more than 35%) retained genes as features. Finally, a 10-fold cross-validation was applied to choose regularization parameters and the final model was fitted using the whole data set before predicting new samples.

We next tested this algorithm by analyzing global gene expression of a patient-derived dermatofibrosarcoma protuberans. The algorithm ranked miR-193a-3p as the most potent miRNA against this tumor, out of four tested cell cycle-targeting miRNAs.[28] Indeed, systemic delivery of miR-193a-3p to mice bearing xenografts of this tumor significantly blunted tumor growth in vivo.[28] These observations suggest that our elastic net regression model can be used to predict the response of patient-derived tumors to cell cycle-targeting miRNAs.

Cell cycle-targeting miRNAs specifically affect tumor growth

To evaluate a therapeutic potential of cell cycle-targeting miRNAs, we systemically administered two cell cycle-targeting miRNAs, miR-193a-3p or miR-214-5p, encapsulated into lipidoid nanoparticles, to seven mouse xenograft models including three treatment-refractory patient-derived tumors.[28] This nanoparticle-based delivery resulted in a significant tumor growth reduction in all of these models, without causing any detectable adverse effects for animals' health.[28] To analyze whether this was due to a preferential uptake of miRNA by tumor cells, we harvested ten organs and tumors from miRNA-treated animals, and analyzed the levels of this miRNA by RT-qPCR. Prior to miRNA-administration, xenografted tumors displayed overall low endogenous levels of cell cycle-targeting miRNAs, as compared to normal organs (Fig. 4A-B). Following miRNA delivery, the most significant miRNA uptake was observed in livers and spleens, whereas the uptake to tumor cells was less pronounced (Fig. 4A-B). Hence, the therapeutic effect of cell cycle-targeting miRNAs cannot be explained by their preferential uptake by tumor cells. We have also determined that, upon systemic miRNA administration in vivo, the reduction of cyclin/CDKs levels in tumor cells was comparable with that in infiltrating normal mouse cells. Likewise, miRNA transfection into in vitro cultured cells caused a similar reduction of cyclin/CDK levels in tumor cells and in non-transformed cells (data not shown). These results indicate that the response of tumor cells to cell cycle-targeting miRNAs is likely not caused by a preferential depletion of cyclins/CDKs in tumor cells. Instead, we propose that healthy cells are better equipped to cope with a reduction in the levels of cell cycle proteins than tumor cells

Discussion

Our recent work[28] along with the data presented here highlights cell cycle-targeting miRNAs as an attractive therapeutic
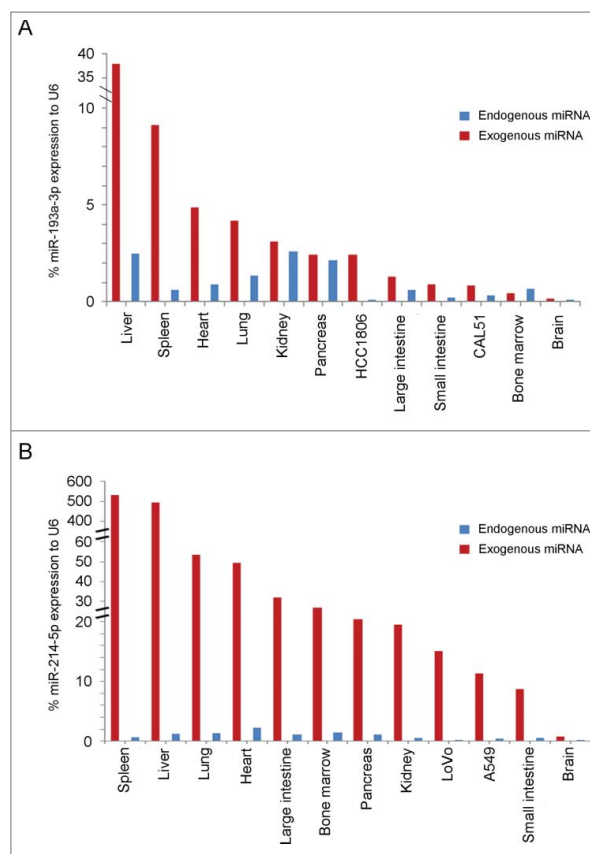

Figure 4. Nanoparticle-mediated miRNA uptake in vivo. (A) Expression levels of endogenous miR-193a-3p (blue bars) and exogenous miR-193a-3p (red bars) in the indicated normal mouse organs and in tumors derived from HCC1806 and CAL51 cells, relative to the ubiquitously expressed and highly abundant small RNA U6. N = at least 3 mice per tumor/organ. This chart was created by re-plotting the data shown in Figures S6A and S6O, Hydbring et al.[28] (B) Expression levels of endogenous miR-214-5p (blue bars) and exogenous miR-214-5p (red bars) in the indicated mouse organs and in tumors derived from LoVo and A549 cells, relative to the ubiquitously expressed and highly abundant small RNA U6. N = at least 3 mice per tumor/organ. Bars displaying endogenous miR-214-5p levels are re-plotted from data shown in Figure S8A, Hydbring et al.[28]

option against aggressive human tumors. Using nanoparticle-based delivery method, we achieved substantial anti-tumor effects with no detectable toxicity against normal organs. We believe our approach for miRNA selection and validation, in particular an unbiased screening strategy, has been essential for the therapeutic success in vivo.

In our approach, we first identified miRNAs that target multiple cyclins and CDKs using genome-wide screens. This potentially could have been done in silico. Indeed, several prediction algorithms are available to identify candidate miRNAs that target a given transcript.[24,42-53] Our systematic miRNA screens confirmed overall a good congruency between predicted versus observed targeting by miRNAs.[28] However, prediction programs often neglect cryptic miRNA binding sites and are therefore less reliable than the biological assays. Indeed, if solely relying on algorithms predicting miRNA targeting, we would not have been able to uncover the full range of cell cycle-targeting miRNAs. Moreover, we followed our screens by profiling selected miRNAs against a panel of human cancer cell lines, and chose the most potent ones for in vivo delivery.

We propose that with proper selection strategy, individual miRNAs may represent very attractive anti-cancer therapeutic compounds. A therapeutic miRNA can be selected based on targeting multiple proteins within the same pathway, in particular in pathways that are essential for cancer cells. If desired, a combination of two or more miRNAs can be used, thereby targeting parallel pathways. Targeting factors in overlapping biological pathways is likely to blunt the tumors' ability to activate compensatory mechanisms and result in a durable biological response. Importantly, tumor-suppressive miRNAs may be downregulated in tumor cells,[25,26] and this may increase tumor cell sensitivity to their expression. Lastly, miRNAs reduce, but not extinguish expression of their targets, and this may offer a therapeutic window of selectively targeting cancer cells. We believe that this likely explains why normal cells can tolerate expression of cell cycle-targeting miRNAs better than cancer cells.

A major challenge in therapeutic application of miRNAs is their limited delivery to solid tumors.[25,26,54,55] However, we achieved significant anti-tumor effects in vivo, despite modest miRNA uptake into tumor cells. We hypothesize that an improved delivery of cell cycle-targeting miRNAs might cause a stronger anti-tumor effect, possibly leading to tumor regression. Indeed, we observed that high-level overexpression of cell cycle-targeting miRNAs in several cultured human cancer cell lines essentially wiped out tumor cells, by triggering tumor cell death.[28] An efficient in vivo administration of miRNAs is difficult due to their size and double-stranded nature, which requires complex delivery vehicles. In our study, miRNAs were encapsulated into lipidoid nanoparticles,[28] originally developed for delivery of siRNAs to hepatocytes.[56] It is likely that customized chemical engineering of nanoparticles, including surface coating using tumor cell- specific ligands,[57-60] will significantly improve miRNA delivery and augment their therapeutic efficacy in vivo.

Materials and methods

Analyses of cell cycle-targeting miRNA levels in mouse liver tumors

Mouse liver tumor nodules and adjacent tissue were dissected from mice 10 weeks post hydrodynamic injection of DNA encoding c-Met and constitutive active β-catenin. Hydrodynamic injections were performed as described previously.[41] RNA was extracted using mirVana miRNA extraction kit (Ambion), according to manufacturers instructions. Quantification of cell cycle-targeting miRNAs was performed by proprietary technology at Firefly Bioworks Inc. Cambridge MA. Signals from quantified miRNAs were normalized to multiple mouse small nuclear RNAs.

Disclosure of potential conflicts of interest

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