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A new plasmid-based microRNA inhibitor system that inhibits microRNA families in transgenic mice and cells: a potential new therapeutic reagent

H Cao1, W Yu2, X Li3, J Wang1, S Gao1, NE Holton3, S Eliason2,4, T Sharp2 and BA Amendt2,3,4

Current tools for the inhibition of microRNA (miR) function are limited to modified antisense oligonucleotides, sponges and decoy RNA molecules and none have been used to understand miR function during development. CRISPR/Cas-mediated deletion of miR sequences within the genome requires multiple chromosomal deletions to remove all functional miR family members because of duplications. Here, we report a novel plasmid-based miR inhibitor system (PMIS) that expresses a new RNA molecule, which inhibits miR family members in cells and mice. The PMIS engineered RNA optimal secondary structure, flanking sequences and specific antisense miR oligonucleotide sequence bind the miR in a stable complex to inhibit miR activity. In cells, one PMIS can effectively inhibit miR family members that share the same seed sequence. The PMIS shows no off-target effects or toxicity and is highly specific for miRs sharing identical seed sequences. Transgenic mice expressing both PMIS-miR-17-18 and PMIS-miR-19-92 show similar phenotypes of miR-17-92-knockout mice. Interestingly, mice only expressing PMIS-miR-17-18 have developmental defects distinct from mice only expressing PMIS-miR-19-92 demonstrating usefulness of the PMIS system to dissect different functions of miRs within clusters. Different PMIS miR inhibitors can be linked together to knock down multiple miRs expressed from different chromosomes. Inhibition of the miR-17-92, miR-106a-363 and miR-106b-25 clusters reveals new mechanisms and developmental defects for these miRs. We report a new tool to dissect the role of miRs in development without genome editing, inhibit miR function in cells and as a potential new therapeutic reagent.

INTRODUCTION

MicroRNAs (miRs) are short noncoding RNA molecules, ~22 nucleotides (nts) long, that regulate messenger RNA (mRNA) transcripts post-transcriptionally through binding to complementary sequences on target mRNA.1–4 The human genome may contain over 1500 miR species (miRBase, release 18) and it has been estimated that more than half of protein coding genes could contain over 1500 miR species (miRBase, release 18) and it has

1Department of Anatomy and Cell Biology, Carver College of Medicine, University of Iowa, Iowa City, IA, USA;
2Department of Anatomy and Cell Biology, Craniofacial Anomalies Research Center, Carver College of Medicine, University of Iowa, Iowa City, IA, USA;
3Dow Institute of Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA, USA and
4Craniofacial Anomalies Research Center, Carver College of Medicine, University of Iowa, Iowa City, IA, USA.

E-mail: brad-amendt@uiowa.edu

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and is a potential therapeutic reagent for cancer and other diseases.

RESULTS

Design and optimization of the PMIS

The PMIS design started with an anti-miR oligodeoxynucleotide-based approach that expressed a nucleic acid sequence that is antisense to the miR.22,30–34 The antagonims used in our study bind to the complete miR sequence including the seed region and flanking sequences to enhance the specificity and binding affinity of the antagonisms. The antagonism sequence was ligated to a custom-designed ~120 nt RNA secondary structure that facilitates its function, stability and processing. The construct is expressed using the U6 Pol III promoter, which does not produce as many transcripts as Pol II promoter (cytomegalovirus) activation. Each nucleotide of the 120-nt backbone RNA structure was selected for its effect on the specificity and mature miR inhibition activity. The initial design began with an 8 nt double-stranded (ds) sequence flanking each end of the antagonist (stem and loop; in blue), 8ds-antiS-8ds (Figure 1a). Multiple constructs were designed with different lengths of each double-stranded region and eight of these are shown and the complete construct is expressed (Figure 1b). The PMIS design started with an anti-miR oligodeoxynucleotide that expressed a nucleic acid sequence that is antisense to the miR.

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**Figure 1.** Optimization of miR inhibitor efficiency. (a) miR inhibitor designs. Black line indicates the specific inhibitor sequence that is complementary to the full-length miR. Blue line indicates the double-stranded (ds) and stem loop RNA. (b) Predication of RNA secondary structure of the final inhibitor design by RNA mfold. The miR antisense sequence is boxed. (c) Structure of the vector construct used to transduce cells, either one or multiple miR inhibitors can be cloned into the vector. (d) Effects of secondary structure length, local AU content and last A ribonucleotide on miR inhibitor efficiency. The psiCHECK2 vector (Ck2) does not contain a miR-17 binding site after Rluc. The Ch2-miR-17 reporter contains a single miR-17 binding site cloned after Rluc. All co-transfections with inhibitors use the Ck2-miR-17 reporter. Normalized Rluc to Fluc ratio of miR inhibitor vector without miR-17 binding site was set as 100%. miR inhibitor designs were co-transfected with miR inhibitor containing a miR-17 binding site into HEK 293FT cells, which endogenously express miR-17. Rluc and Fluc activity was measured 48 h after transfection. The sponge plasmid has six tandem miR-17 binding sites. T-test was performed against the sample, marked by an arrow. *P < 0.05 and **P < 0.01. CMV, cytomegalovirus; EGFP, enhanced green fluorescent protein; HSV, herpes simplex virus.
PMIS-miR-200c does not change the level of miR-17 (Figure 2a). PMIS-miR-200c inhibits miR-200c to ~90% but does not affect miR-17 levels (Figure 2a). The reduction of miR levels using a sponge miR inhibitor was previously reported to be an artifact of miR degradation by inhibitors.36,37 As controls, the levels of miR-17 and miR-200c were reduced by PMIS-miR-17-18 and this is consistent with other studies demonstrating miR degradation by inhibitors.36,37 As controls, the levels of miR-23a, miR-200b and miR-19 were not affected by PMIS-miR-17-18. We also wanted to determine if the PMIS vector and/or PMIS-miR-17-18 construct were degraded or cleaved in the cells; however, both were expressed and not cleaved by Dicer (Figure 2b). However, it is also likely that the PMIS system can inhibit miR activity by sequestration and slow degradation of the miR as we detect low levels of miR expression.24,29 Bim expression, a pro-apoptotic gene involved in B-cell development and a known target of miR-17,38 was elevated in cells transfected with PMIS-miR-17 compared with cells with empty vector and PMIS-miR-200a (Figure 2c). Furthermore, Bim transcripts levels were increased ~3-fold in PMIS-miR-17 cells (data not shown). PTEN (phosphatase and tensin homolog) was identified in a screen for genes regulated by miR-200a/141 and this is consistent with other previous data that an miR inhibitor could inhibit several miRs that share the same seed sequence.39,40 PMIS for miR-200b, -200c or -429 inhibited the miR-200b/200c/429 subfamily efficiently but not the miR-200a/141 subfamily (Figures 4b and c). This result is consistent with other previous data that an miR inhibitor could inhibit several miRs that share the same seed sequence.39,40

miR-200 family knockdown by a single miR inhibitor plasmid

Because one miR inhibitor can potentially inhibit different members of the same miR family, we asked if a PMIS construct containing two inhibitors targeting an miR family could inhibit the complete miR family. To test this, cells were transfected with the miR reporter luciferase construct, the associated miR that binds to the target in the luciferase construct and the miR inhibitors or empty vector. The miR-200 family has two subfamilies, miR-200a-3p/141-3p and miR-200b-3p/200c-3p/429-3p (Figure 4a). These two subfamilies have one nucleotide difference (underlined) in their seed regions. PMIS constructs for miR-200a or -141 (PMIS-miR-200a or PMIS-miR-141) inhibited the miR-200a/141 subfamily but not the miR-200b/200c/429 subfamily (Figures 4b and c). This result is consistent with other previous data that an miR inhibitor could inhibit several miRs that share the same seed sequence.39,40

Figure 2. Efficiency of the PMIS. (a) PMIS-miR inhibitors reduce specific miR expression. RT-PCR of mature endogenous miR-17 and miR-200c from 293 cells transfected with the indicated PMIS inhibitor. (b) Northern blot of mature endogenous miRs and the PMIS construct from 293 cells transfected with PMIS vector or PMIS-miR-17-18. U6 RNA is shown as a loading control. (c) Western blot of Bim, a known target of miR-17 from cells transfected with the PMIS vector, PMIS-miR-17 or PMIS-miR-200a. In addition, a western blot of PTEN, identified as a target of miR-17. β-Tubulin and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) are shown as loading controls.
them for functionality. To test whether the PMIS inhibits noncanonical miRs, inhibitors were made to two miRs that do not require Drosha (miR-877-5p and miR-1224-5p) and one that does not require Dicer (miR-451a). The miR inhibitors inhibited both miRs (Figure 5a), small interfering RNAs (siRNAs) are different from miRs in that they do not require Drosha and Dicer processing for function. However, both siRNAs and miRs load into a functional RNA-induced silencing complex (RISC). To test whether the PMIS also works on siRNAs, we designed an siRNA to luciferase mRNA. For function. However, both siRNAs and miRs load into a functional RNA-induced silencing complex (RISC). To test whether the PMIS also works on siRNAs, we designed an siRNA to luciferase mRNA. The PMIS to this siRNA efficiently blocks siRNA function (Figure 5a). As a negative control, this PMIS does not knock down luciferase activity by itself (Figure 5b). Thus, the PMIS works specifically on all miRs and siRNAs.

The BMP2R gene was identified as a target of miR-17 in PMIS-miR-17-18 bioinformatics analyses of transgenic mouse tissues. To demonstrate that PMIS-miR-17-18 can also inhibit endogenous miR-17 from repressing BMP2R expression, the BMP2R 3′UTR (untranslated region) was cloned into the luciferase vector and co-transfected with miR-17 or vector only and PMIS-miR-17-18 or vector only. PMIS-miR-17-18 caused a twofold increase in luciferase activity, whereas miR-17 overexpression decreased luciferase activity 60% compared with controls (Figure 5c).

miR inhibitor associates with Dicer and argonaute

To determine whether the PMIS constructs were bound by argonaute (AGO), 293 cells were transfected with PMIS vector only, and PMIS-miR-200c or PMIS-miR-17 were subjected to immunoprecipitation (IP) assays using either immunoglobulin G (control) or AGO antibodies. After IP, the complexes were washed and denatured and PCR was performed using primers for PMIS-miR-17. The complexes containing endogenous miR-17 and PMIS-miR-17 were pulled down by the AGO antibody (Figure 6a, lane 6). A similar IP used the Dicer antibody to pull down the inhibitor complex and Dicer was associated with PMIS-miR-17 (Figure 6b, lane 6). In addition, proteins were pulled down that associated with the miR inhibitor using biotin in vitro transcribed RNA and incubated with cell lysates and probed for Dicer and AGO binding by western blot (Figure 6c). The PMIS associates with Dicer and AGO and this association decreased when the miR inhibitor lost its double-stranded RNA structure (Figure 6d). The PMIS double-stranded RNA stem structure (5′ end) appears to bind Dicer; however, because of its short length 18 nts, Dicer does not cleave it as Dicer cleaves 22–24 nt long double-stranded RNA sequences. This lack of PMIS cleavage was also shown by Northern blot in Figure 2. Furthermore, Dicer recognizes the PMIS double-stranded stem structure but not the PMIS-miR interaction, due to the 4 nts mismatch in the antisense oligo design.

To test whether Dicer was required for PMIS function, siRNA was used to knock down Dicer (Figure 6e). The PMIS inhibits siRNA, which targets the luciferase gene (Rluc) and siGFP has no effect, used as a control (Figure 6f). However, after knocking down Dicer with siRNA the miR inhibitor to siRNA-Rluc has no effect on inhibiting the activity of the siRNA to Rluc (Figure 6f).

The PMIS is relatively stable in cells

To determine the relative stability of the miR inhibitor, cells were transfected with lentivirus expressing PMIS-miR-200b. The cells were seeded at identical densities into 6-well plates, and after 24 h, they were subjected to actinomycin D (5 ng/ml) treatment for the indicated time (days). The PMIS showed no decrease in transcript stability after 7 days of treatment, compared with β-actin (Figure 7, top panel). However, 28 S, 18 S and 5 S RNAs were all degraded after 5 days of treatment with actinomycin D (Figure 7, bottom panel). Because the average miR half-life is ~5 days, the PMIS appears to be more stable than miRs, allowing them to remain functional in the cell.42 The actinomycin D-treated cells were dead or dying after 4–5 days and RNA analyzed after 5 days was from dead or dying cells left on the plates. Furthermore, because a single miR can recycle to process multiple mRNA

Figure 3. Specificity of miR inhibitors. (a) Sequence of miR-17 inhibitor and specific mutations to test function. The numbers indicate the mutated ribonucleotide (bold and in caps) and the AUCA sequence in the middle of the inhibitor sequence eliminates a perfect match.27 The seed sequence of the mature miR-17-5p is in bold as is the antisense sequence to the seed sequence in PMIS-miR-17. (b) Ribonucleotide mutation effects on miR inhibitor efficiency. Normalized Rluc to Fluc ratio of miR reporter vector without miR-17 binding site was set as 100%. The PMIS-miR-17 inhibitor mutants were co-transfected with miR reporter vector containing a miR-17 binding site into HEK 293FT cells. Rluc and Fluc activity was measured 48 h after transfection. T-test was performed against the sample, marked by arrow. *P < 0.05 and **P < 0.01.
transcripts, the PMIS stability may facilitate longer interactions with target miRs and provide more efficient inhibition of their activity.37

Analyses of gene regulation by PMIS-miR-17
To evaluate PMIS efficacy and specificity in a systematic analysis, we profiled whole-genome mRNA expression changes after transduction with PMIS-miR-17 in 293 cells. A schematic of the experimental design is shown (Figure 8a). The miR-17 targets were separated into two groups based on TargetScan prediction score, one with a context+ score $< -0.30$ and the other with a context+ score $> -0.30$. Distributions of changes (0.1 unit bins) for mRNA UTRs containing no site (black line), site with a score $> -0.30$ (red line) and site with a score $< -0.30$ (blue line) shows mRNA targets of miR-17 are upregulated in PMIS-miR-17 transfected cells (Figures 8b and c). Upregulation of mRNAs with context+ score $< -0.30$ was significantly more than that from mRNAs with no site ($P < 10^{-13}$, one-sided Kolmogorov–Smirnov (K-S) test). Comparisons between mRNAs with context+ score $< -0.30$ versus context + score $> -0.30$; context+ score $> -0.30$ versus no site were also significant ($P < 10^{-8}$ and $P < 10^{-3}$, respectively) (Figures 8b and c). These results demonstrate that PMIS-miR-17 specifically and effectively inhibit miR-17, which in turn results in the increase in transcripts targeted by miR-17.

![Figure 4](image)

Figure 4. Knockdown of the miR-200 family with a single plasmid. (a) Sequence of mature miR-200 family members. The underlined nucleotide indicates the different ribonucleotide between two subfamilies in the seed region (bold). (b) Inhibition of miR-200a-3p with different PMIS-miR inhibitors. PMIS empty vector or various PMIS-miR inhibitors were co-transfected with miR-200a expression plasmid and miR reporter vector containing a miR-200a binding site into HEK 293FT cell. Rluc and Fluc activity was measured 48 h after transfection. (c) Inhibition of miR-141-3p with different PMIS-miR inhibitors. (d) Inhibition of miR-200b-3p with different PMIS-miR inhibitors. (e) Inhibition of miR-200c-3p with different PMIS-miR inhibitors. (f) Inhibition of miR-429-3p with different PMIS-miR inhibitors. A single plasmid expressing both PMIS-miR-200a-200b inhibits all five members of the miR-200 family. The miR-17 reporter was used as a control. T-test was performed against the sample, marked by arrow. *$P < 0.05$.

To determine if PMIS-miR-17 inhibited other miRs in cells, 293 cells were transduced with the lentiviral PMIS-miR-17 and randomly selected miRs were profiled by real-time PCR (q-PCR) (Taqman probes; Applied Biosystems, Waltham, MA, USA). miR-17 was specific for the inhibition of miR-17-5p and miR-106b-5p (Figure 8d).

PMIS inhibits miRs during mouse development
The PMIS was designed as a pronuclei-injected construct to create transgenic mice that stably express the miR inhibitors. Because the miR-17-92 intragenic cluster has been conventionally deleted, we compared our PMIS-miR-17 to the published miR-17-92 knockout.26,43–45 However, the conventional miR-17-92 knockout does not delete the other miR family clusters, miR-106a-363 and miR-106b-25 (Figure 9a). We made two transgenic mice to inhibit all three clusters. Because the miR-17-92null knockout mice are perinatal lethal, two PMIS constructs PMIS-miR-17-18-19-92 and PMIS-miR-19-92 were pronuclei injected to make transgenic (TG) mice (Figure 9b, underlined sequence). These four miR inhibitors target the four seed regions shared among the miR families (Figures 9a and b). Each PMIS transgene was designed with an antirepressor element upstream of the mouse U6 promoter followed by an miR inhibitor, a second U6
promoter followed by different miR inhibitor, a scaffold-attached region (SAR) and polyA site (Figure 9c).

Multiple founders were generated and PMIS-miR-17-18 no. 6 demonstrated 95% knockdown of miR-17 and 86% knockdown of miR-18a by RT-PCR (Figure 10a). Multiple founders for PMIS-miR-19-92 showed decreased miR expression, PMIS-miR-19-92 no. 7 inhibited miR-19a at 95% and miR-92a at ~65% (Figure 10b). Founders PMIS-miR-17-18 no. 6 and PMIS-miR-19-92 no. 7 were established and these mice were crossed to generate the PMIS-miR-17-18-19-92 TG mice. The PMIS-miR-17-18 and PMIS-miR-19-92 mice are viable; however, ~25% have craniofacial defects that cause neonatal lethality. The PMIS-miR-17-18-19-92 mice are perinatal lethal similar to the miR-17-92\textsuperscript{null} mice. PMIS-miR-17-18-19-92 embryos (E18.5) are smaller than wild-type (WT) embryos with reduced body weight as reported for the miR-17-92\textsuperscript{null} mice (Figures 10c and d).\textsuperscript{30,44} These mice also have small lungs (Figure 10e). Quantitative PCR of PMIS expression in two different PMIS transgenic embryos show high levels of PMIS-miR-17-18 and PMIS-miR-200b, respectively (Figure 10f). Because the WT mice do not express the PMIS, the fold change is compared with no expression. In Northern blots showing PMIS expression, the levels of transcripts are similar to miRs (see Figure 2).

miR-17-92 expression is inhibited in the PMIS-miR-17-18-19-92 mice. Mouse tissues from E18.5 PMIS-miR-17-18, PMIS-miR-19-92 and PMIS-miR-17-18-19-92 TG embryos were analyzed for miR expression. To analyze large miR sets, the Qiagen protocol for miR detection was used to determine miR expression profiles. Specific miR expression associated with PMIS-miR-17-18 and PMIS-miR-19-92 mice were decreased and the complete miR-17-92 family was inhibited in the PMIS-miR-17-18-19-92 embryos (Figure 11a). Furthermore, miR-92a-1-5p, which is not targeted by the inhibitor system, have normal expression level in TG mice. Northern blots of RNA from PMIS-miR-17-18 embryonic mandibles demonstrate specific reduction of miR-17, while miR-92 was not affected compared with WT (Figure 11b). miR-19 expression was decreased in PMIS-miR-17-18-92 embryos but not in WT or PMIS-miR-17-18 (Figure 11b). Both miR-17 and miR-19 expression levels were decreased in the PMIS-miR-17-18-19-92 embryos compared with WT (Figure 11b).

To control for toxicity and nonspecific effects of the PMIS inhibitors, total RNA was isolated from P14 mice livers and probed for specific gene transcripts and miR expression levels. Ndrg3 (N-myc downstream regulated gene 3), Bckdk (branched-chain α-ketoacid dehydrogenase kinase) and Cd320 (Cd320 antigen, putative VLDL receptor), which are also known targets of miR-122,\textsuperscript{46} were analyzed for their expression in WT, PMIS-miR-17-18-92 and PMIS-miR-200a mice. All three genes, which are associated with liver disease and toxicity, were not affected by expression of the PMIS inhibitors (Figure 11c). miR-122 is associated with liver homeostasis and decreased levels of miR-122 are seen in hepatocarcinogenesis.\textsuperscript{47} As expected in the PMIS-miR-17-18-19-92 mice, liver miR-17

Figure 5. Efficiency of PMIS-miR inhibitor targeting noncanonical miRs. (a) Efficiency of PMIS-miR inhibitors targeting intronic miRs that bypass Drosha processing miR-877-5p and miR-1224-5p. Dicer-independent miR-451a and in vitro transcribed siRNA PMIS-miR inhibitor targeting Rluc mRNA served as a control. Rluc and Fluc were assayed as in previous figures. (b) As an additional control, PMIS-anti-Rluc had no effect on luciferase expression and activity. (c) The BMPR2 3′UTR was cloned into the luciferase vector and co-transfected with PMIS-miR-17-18, vector only or pSil-miR-17 (overexpression) in 293 cells.

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Figure 6. miR inhibitor interaction with RISC. (a) Immunoprecipitation (IP) of miR inhibitor using AGO2 antibody. PCR primers were used to detect PMIS-miR-17 after pull down, and U6 was used as loading control. Immunoglobulin G antisera was used as a control and PMIS-miR-200c transfected as a PCR control. (b) IP of miR inhibitor using a Dicer antibody. Controls were as in panel a. (c) Schematic of the Dicer and Ago2 pull-down assay. (d) Pull down of AGO2 and Dicer with biotinylated PMIS-miR-17. Biotinylated PMIS-miR-17 RNA or PMIS-miR-17 RNA without the double-stranded regions were incubated with 293 cell extracts, washed, denatured and analyzed by polyacrylamide gel electrophoresis (PAGE). Western blot was performed using Dicer and AGO antibodies. Proteins were detected using ECL reagents (GE Healthcare). (e) Western blot of Dicer after knockdown of Dicer with siRNA. Cell lysates (15 μg) were resolved by PAGE after transfection with siGFP, siDicer 1-1 and siDicer 1-2 (two different siRNA constructs) or water as a control. Western blot was performed using Dicer antibody and β-tubulin antibody as a loading control. (f) Knockdown of Dicer impaired miR inhibitor function. Rluc vector and in vitro transcribed siRNA targeting Rluc was co-transfected with miR inhibitor to siRNA-Rluc and in vitro transcribed siRNA targeting GFP or two different sites of the Dicer transcript. Rluc and Fluc activity was measured 48 h after transfection.

Figure 7. PMIS-miR inhibitors are relatively stable in the cell. Cells stably expressing PMIS-miR-200b were seeded at identical concentrations into six-well plates and subjected to actinomycin D treatment for 7 days. After 1, 3, 5 and 7 days, cells were harvested, total RNA harvested and PMIS-miR-200b was analyzed by RT-PCR (top panel). β-Actin was used as a control. The bottom panel shows the 28S, 18S and 5S RNAs as controls. Another control contained the isolated total RNA without reverse transcription (no RT control) used in the PCR reaction to detect possible PMIS DNA; however, no PMIS DNA was detected in the samples.
Craniofacial defects in the PMIS-miR-17, PMIS-miR-17-18-19-92 mice as well (data not shown). However, several mice also showed a lack of or incomplete palate shelf fusion with the nasal septum (Figure 12).

Skeletal defects are associated with disrupted miR-17 activity. The PMIS-miR-17-18-19-92 mice (E18.5) have a cleft palate as shown for the miR-17-92null mouse. In the miR-17-92null the palate shelves elevate and adhere with the nasal septum but do not form the secondary palate and this was observed in the P0 PMIS-miR-17-18-19-92 mice as well (data not shown). However, several mice also showed a lack of or incomplete palate shelf fusion with the nasal septum (Figure 12).

A unique aspect of the PMIS is its ability to target specific miRs within a cluster. Analyses of PMIS-miR-17-18-19-92 mice show that from mRNAs with no site, context+ score −0.30 versus context+ score −0.30 was significantly more than that from mRNAs with context+ score −0.30 and the miR-17-92null mice show no toxicity. The PMIS-miR-17-18-19-92 and PMIS-miR-200a mice do not develop cancer or die due to liver toxicity or toxicity problems.
miRs are essential regulators of gene expression and critical for normal embryonic development, tissue morphogenesis, cellular functions such as metabolism and are associated with cancer cell types. However, our knowledge of the full aspects of miR function during development remains fragmentary because of the limited availability of experimental tools to knock down or knock out miRs in vivo. Several approaches have been reported to knock down miRs in adult mice but not during embryonic development. One method reported using the Tough Decoy system delivered using recombinant adeno-associated virus injected into adult mice.50 The recombinant adeno-associated virus expressing anti-miR-122 Tough Decoy reduced serum cholesterol by >30% for 25 weeks in mice. A second method used lentiviral vectors to express miRT sequences (sponges, decoys, antagomirs) to miR-233 in transduced bone marrow stem and progenitor cells transplanted into mice recipients.51 Although both systems inhibited miR activity for their specific miR target, there are limitations to both systems. Furthermore, both methods have dose–response problems and neither have the ability to function as a transgene for developmental studies and long-term knockdown of multiple miR family members. A third method used a transgenic mouse expressing a sponge to bind miR-183 and these mice demonstrated retina defects.52 These methods are critically advancing our knowledge of miR function in vivo and provide a wealth of knowledge on miR function.

New miR inhibitor design and function

Our findings advance these methods by using a novel RNA structure that inhibits miR function in vivo and can target multiple miRs with the same seed sequence. The PMIS system is more efficient than the sponge or antagomir sequences. The PMIS was designed independently of the Tough Decoy system50 and incorporates a unique RNA secondary structure that facilitates in vivo anti-miR function. The PMIS has an anti-miR binding sequence including a four nucleotide bulge region. We demonstrate that both Ago and Dicer bind to the PMIS and we speculate that these interactions stabilize the miR–inhibitor complex. Dicer is not required for RISC loading in mammals but its interaction with the PMIS may facilitate the inhibitor’s function.53 Previous studies have indicated that overexpression of shRNAs and RNAi hairpin structures could titrate the miR processing machinery and lead to nonspecific effects and toxicity.54,55,56 We observe no toxic effects or nonspecific dysregulation of miRs in PMIS-expressing cells or in...
over 500 mice expressing different miR inhibitors. Each PMIS-miR inhibitor mouse line has specific defects associated with the expression pattern of the specific miR.

We propose a model for PMIS functional inhibition of miR activity (Figure 14). After miR biogenesis, the mature miR can remain associated with Dicer, Ago2, TRBP (transactivating response RNA-binding protein) and PACT from the RISC and facilitate mRNA target recognition. Dicer associates with TRBP and Ago2 to facilitate transfer of the miR to the RISC. Dicer and Ago2 and other proteins in the complex facilitate binding of the mature miR to the PMIS in a stable complex. Dicer transiently associates with the PMIS double-stranded stem structure and recycles owing to the short-stem length, which it cannot cleave. The PMIS-miR complex is stable in the cell and leads to degradation of the miR, processed by as of yet an unknown factor.

Advantages of the PMIS to analyze miR function and identification of developmental defects

We demonstrate an effective and specific system to inhibit miRs and miR clusters in mice using a transgenic approach. Transgenic mice expressing both PMIS-miR-17-18 and PMIS-miR-19-92 have a similar phenotype as the miR-17-92 knockout mice, demonstrating the efficiency and specificity of the PMIS system in vivo. Furthermore, the PMIS can dissect the function of specific miRs in the miR-17-92 clusters, which is an advantage over traditional genomic knockout approaches. We have demonstrated that PMIS-miR-19-92 and PMIS-miR-17-19-92 mice have thyroid developmental defects. These data correlate with report showing that miRs in the miR-17-92 cluster were overexpressed in thyroid cancer cells compared with primary thyrocytes. Furthermore, by inhibiting specific miRs in the cluster different effects on growth, apoptosis and cellular senescence were observed in these thyroid cancer cells. By inhibiting the miR-19-92 family, we show that these miRs are required for thyroid development, and inhibition of the complete family of miR-17-92 causes more severe defects including bone, cervical and craniofacial defects.

Utility of the PMIS

Compared with genomic knockouts, sponges and other approaches, making and using the PMIS inhibitors is simple, fast, cost effective and non-toxic. It can be used in viral systems to create stable PMIS-expressing cells and tissues in culture, or in transgenic mice to study miR function. The PMIS as a plasmid can be delivered using nanoparticles, viruses and lipid-based systems to treat cancers with aberrant miR expression or to protect cells and tissues from aberrant gene expression. There is great utility for this system as a therapeutic reagent as the PMIS is extremely specific for each miR. Because this is a plasmid-based miR inhibitor, it is easy and inexpensive to use without reapplication in cells that is required for other modified antisense oligonucleotide systems.

MATERIALS AND METHODS

Animals

All animals were housed at the University of Iowa, in the Program of Animal Resources and were handled in accordance with the principles and procedure of the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the University of Iowa IACUC guidelines. PMIS-miR DNA was excised from the plasmid and used for pronuclear injection. Donor female mice (FVB/NCr), stud male (FVB/NCr), vasectomized male (ICR) and recipient female (ICR) were used in the
experiments. Multiple founders (eight for each construct) were analyzed for transgene expression and crossed to BALB/c mice and re-evaluated for expression. Observation of a vaginal plug was counted as embryonic (E) day 0.5, and embryos and neonates were collected at various times. miR expression in transgenic mice. Sponge reporter was made by inserting six tandem binding sites for miR-17 into the psiCHECK2 vector.

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**Figure 11.** miR expression levels in PMIS-miR-17-18, PMIS-miR-19-92 and PMIS-miR-17-18-19-92 TG mice. (a) Total RNA was harvested from E18.5 mice heads and subjected to miR expression analyses as per the manufacturer’s instruction (Qiagen, Hilden, Germany). miR expression is calculated as $\Delta \Delta CT$ after normalization to control primers. All data was compared with WT mice ($N=2$), two different mice and qPCR performed in triplicate for each mouse; *P < 0.05). (b) Northern blot analyses of miR-19 and miR-17 expression in total RNA harvested from E18.5 mouse mandibles. PMIS-miR-17-18 mouse demonstrated reduce miR-17; PMIS-miR-19-92 mouse showed reduced miR-19 and the PMIS-miR-17-18-19-92 mouse had reduced expression of both miR-17 and 19. (c) qPCR of Ndrg3, Bckdk and Cd320 transcripts in total RNA isolated from PMIS-miR-17-18-19-92, PMIS-miR-200a or WT E18.5 mouse liver ($N=2$). (d) miR expression in RNA isolated from PMIS-miR-17-18-19-92, PMIS-miR-200a or WT E18.5 mouse liver.

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Construction of miR reporter, expression and inhibitor plasmids

To anneal oligos, oligos were heated at 70 °C for 10 min and then slowly cooled to room temperature. For ligation, annealed oligos were phosphorylated by T4 polynucleotide kinase (NEB) first and then ligated into vectors using the Quick Ligation Kit (Roche, Mannheim, Germany) as per the instructions. To construct miR reporters, one perfect match miR binding site was ligated after the Renilla luciferase gene in psiCHECK2 (ck2) vector (Promega dual reporter system, Madison, WI, USA) digested with NotI and Xhol. For example, to construct the miR reporter for miR-17, two short oligos: miR-17 fl, 5’-TCGAAATGACCCTACCTGACGTGTAAGACGACATTGTGCTGAGGCTGC-3’ and miR-17 rt, 5’-GGCCGCCTAGTCAGGAGTTAAGTCACGGGAAAGTACTGAGCGGCATGAGCTGC-3’ were annealed and cloned into psiCHECK2 vector. The following oligos were also used: miR-200a fl, 5’-TCGAATGACCTACGTTGAATAACTGTCTGGTAAAGATGGGGTCAT-3’; miR-200a rt, 5’-GGCCGCAGCTCGAGTAACACTGTCTGGTAACGATGTGGTCA-3’. To link two different miR inhibitors, one plasmid is digested by XhoI and BsmBI sites in the most effective inhibitor design. Ascl and Pmel sites were inserted between ApaI and Bsal sites before the U6 promoter. A Smal site was inserted before Xhol after the Pol III terminator. This vector is termed pmiRI for plasmid of miR inhibitor. After digestion by BsmBI, pmiR can be used to clone different miR inhibitors into it after annealing and ligation of different miR binding sites with a central bulge. To link two different miR inhibitors, one plasmid is digested by Ascl and Smal to release a fragment of ~500 bp; this fragment can be ligated into another miR inhibitor digested by Ascl and Pmel. Plasmid constructs are shown in Figure 1c for cell transductions and Supplementary Figure 5 for transgenic mice. Sponge reporter was made by inserting six tandem binding sites for miR-17 into the psiCHECK2 vector.
Cell culture, transfection and luciferase assays
HEK 293FT (Invitrogen, Waltham, MA, USA) and MDCK (ATCC, Manassas, VA, USA) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. HEK 293FT cells are plated the day before transfection. Twenty nanograms of miR reporter and 200 ng of miR inhibitor are co-transfected into each well of 12-well plates using Fugene6 (Roche). For exogenous miR expression, 200 ng miR expression plasmid was transfected into the cells. The Dual-Luciferase Reporter assay (Promega, Madison, WI, USA) was performed 48 h after transfection as per the instructions. siRNA targeting Dicer (5′-AAGGACGGUGUUCUUGGUCAAC-3′ and 5′-CUGCUCUUGAAGCAGCUUGGAUC-3′) and GFP (5′-AGGACGACGGCAACUACAAGAC-3′) were in vitro transcribed and transfected into cells as described.57

Stability assay of the PMIS constructs
MG-63 cells were transduced with PMIS-miR-200b lentivirus, and after 48 h, cells were seeded in 6-well plates at the same density (30–40%) with 0.1% dimethyl sulfoxide in Dulbecco’s modified Eagle’s medium. After 24 h, cells are treated with actinomycin D at the concentration of 5 ng ml⁻¹ (marking the time point as day 0). Cells were harvested at different time points and total RNA was isolated using Qiagen miRNeasy Mini Kit (Qiagen). cDNAs were made by using TaKaRa PrimeScript RT Master Mix Kit (Hilden, Germany) The PCR cycles were as follows: initial denaturation at 95 °C for 1 min, 94 °C for 30 s, annealing at 60 °C for 30 s and extending at 72 °C for 30 s. The PCR primers used were as follows: human β-actin primers, 5′-CATGTACGTTGCTATCCAGGC-3′ and 5′-CTCCTTAATGTCACGCACGAT-3′; PMIS-miR-200b primers, 5′-CTAATCATCATTACCAATCAGACAGTATTA-3′ and 5′-GTCAGCTCTTAGTATTCATGAGATG-3′. Total RNA from each time point and PCR products were detected in agarose gels. All PCR products were confirmed by sequencing.

Lentivirus production and transduction
For lentivirus production, 6-cm dish of HEK 293FT cell were transfected with 2.8 μg of psPAX2, 1.9 μg of pMD2.G and 4.5 μg of miR inhibitor or control plasmid using Fugene HD (Roche). Supernatants were collected and passed through 0.45 μm filter 28 h after transfection. To concentrate virus preparations, 40 ml of supernatant was centrifuged at 26 000 r.p.m. for 2 h at 4 °C. Pellets were resuspended in 100 μl of Dulbecco’s modified Eagle’s medium at 4 °C overnight. For lentivirus transduction of MDCK cells, 1 x 10⁶ cells were seeded in 6-cm dish immediately before the addition of 30 μl of concentrated virus. MG-63 cells used for actinomycin D experiments were subcultured and transferred to 6-cm dishes at 30% confluence. Virus was added immediately after plating and cultured for 2 weeks with media change every 2–3 days. Puromycin was added for

Figure 12. Craniofacial and bone defects in PMIS-miR-17-18, PMIS-miR-19-92 and PMIS-miR-17-18-19-92 embryos. E18.5 embryos were harvested and heads were stained with Alcian blue and Alizarin red to detect cartilage and bone, respectively. Cross-lines were set to WT size limits and copied onto mutant heads. PMIS-miR-17-18 embryos have a reduced cranial width compared with WT but normal ossification. PMIS-miR-17-18-19-92 embryos show reduced width and anterior–posterior length but normal ossification. However, PMIS-miR-17-18-19-92 embryos have reduced anterior–posterior length, reduced cranial width and a delay in ossification of the interparietal (IP) bone. A cleft palate was identified in PMIS-miR-17-18-19-92 E18.5 embryos, coronal sections, hematoxylin and eosin (H&E) staining (bottom panels, arrows).
selection and stable PMIS-miR-17-expressing cells were used to analyze the stability of the PMIS.

Fluorescence-activated cell sorting
Flow cytometry facility of the University of Iowa preformed cells sorting using the Becton Dickinson Aria II (Franklin Lakes, NJ, USA). Cell suspensions were placed in a 12 × 75 mm² test tube with the final cell concentration between 5 × 10⁶ and 30 × 10⁶ cells per ml. Before sorting, cells are filtered through 70 μm nylon mesh (Falcon 352350, Bedford, MA, USA). Cells are then sorted by GFP and collected at 37 °C into 12 × 75 mm² test tube in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The cells are then cultured.

RT-PCR, western blot and immunofluorescence
For quantification of mature miRs, Taqman probe (Applied Biosystems) were used as per the instructions. Small RNA U6B served as a control. For quantification of miR primary transcripts, iQ SYBR Green Real-Time PCR Supermix (Bio-Rad, Hercules, CA, USA) was used as per the manufacturer’s instructions. β-Actin mRNA served as a control. For western blots, ~20 μg of cell lysates were loaded and ran in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF filters (Millipore, Darmstadt, Germany), immunoblotted using antibodies and detected by ECL reagents (GE Healthcare, Chicago, IL, USA). The dilution of Dicer antibody (Abcam, Cambridge, MA, USA) was 1:500. AGO2 antibody (Cell Signaling, Danvers, MA, USA) was 1:1000. For immunofluorescence, cells were fixed by cold acetone, blocked with 10% serum of secondary antibody, incubated with primary antibody for 1 h and detected by secondary antibody fluorescence. Cells were counterstained with DAPI (4',6-diamidino-2-phenylindole).

RT-PCR of mature miRs from E18.5 mouse heads
E18.5 embryos were harvested and heads were used for total RNA isolation. Total RNA including miR from mouse tissues was prepared using the miRNeasy Mini Kit (Qiagen). qPCR analysis of selected miRs was performed using TaqMan miR assay probes (Applied Biosystems), and U6B

Figure 13. Skeletal defects in the PMIS-miR-17-18-19-92 embryos. E18.5 embryos were harvested and bodies were stained with Alcian blue and Alizarin red to detect cartilage and bone, respectively. (a) PMIS-miR-17-18-19-92 embryos show delayed overall ossification, lack of thyroid structures (black arrow) and a loss of the processus angularis (circle) and microcephaly. (B) The PMIS-miR-17-18 mice have normal thyroid development and structures, but the first cervical (C1) vertebra is malformed. The PMIS-miR-19-92 mice have lost the thyroid structures (black arrow), and have a malformed C1 vertebra. The PMIS-miR-17-18-19-92 mice are defective for multiple cartilages and thyroid structures.
Immobilization of RNA on agarose beads and pull-down assay

miR inhibitor RNA with and without flanking double-stranded RNA was synthesized in vitro by T7 RNA polymerase and DNA oligonucleotide templates. These RNA were biotinylated using 3′-End Biotinylation Kit (Thermo Scientific, Waltham, MA, USA). Biotinylated RNAs were incubated with streptavidin-conjugated agarose beads for 1 h at 4 °C. HEK 293FT cell were collected and sonicated with protease inhibitor on ice. The cell lysate were preclared with beads only. After preclearing, cell lysates were incubated with RNA immobilized on the streptavidin beads and rotated for 12 h at 4 °C. After washing six times, proteins associated with the beads were released from the beads by boiling. Proteins were analyzed by western blot. A protocol that was previously reported was used for PMIS pull down. Briefly, miR inhibitor of miR-17 with and without flanking double strand was produced by in vitro transcription. For AGO2 and DICER pull down, cell extract of 293FT cell stably expressing PMIS vector, PMIS-miR-200c or PMIS-miR-17 were incubated with AGO2 (Cell Signaling) and DICER (Abcam) antibodies. RNAs were eluted from the pull-down complex and primers for PMIS-miR-17 and U6 were used to carry out RT-PCR.

mRNA microarray, correlations of mRNA fold change with miR binding site

Total RNA was extracted from 293FT cells stably expressing PMIS vector or PMIS-miR-17. Human Genome microarray hybridization and scan were performed at the Texas A&M University Genomics Core. Microarray data were analyzed with R. Microarray signal intensity were normalized by global median and log2 transformed. We grouped 22k mRNA (genes) into three groups, 20 K mRNA without miR-17 binding site (predicted with TargetScan), 2 K mRNA with miR-17 binding site that have context score larger than −0.3 (a score TargetScan used to measure how likely miR can inhibit target mRNA, the lower the score the more likely), another 277 mRNA with miR-17 binding site that have context score less than −0.3. Distribution of mRNA fold change (PMIS-miR-17 vs PMIS vector) of these three groups was plotted with R using different colors. One-sided K–S test were used to calculate P-value for each pair of comparison between these three groups.

Skeletal preparations

E18.5 embryos were harvested and placed on ice for 20 min and then scalded the embryos in hot tap water (65 °C) for 30 s. After skin removal, the embryos were fixed in 95% ethanol overnight. Embryos were incubated in Alcian blue solution (0.015% Alcian blue BGX in 1:1 mixture of acetic acid and 95% ethanol) and Alizarin red solution (0.005% Alizarin red in 1% KOH) for cartilage and bone staining. Extra tissues were removed in 2% KOH, skeletons were cleared in glycerol-KOH solution (1% KOH, 20% glycerol) and Alizarin red solution (0.005% Alizarin red in 1% KOH) for comparison between different genotype of embryos.

Figure 14. Working model for inhibitor complex. The mature miR is processed and associated with proteins that facilitate its interaction into an RNA-induced silencing complex with the mRNA or the PMIS. The PMIS transiently binds these factors in association with the mature miR. Dicer is associated with AGO2 and TRBP to facilitate transfer of the miR to the PMIS. However, Dicer is released as it cannot cleave the PMIS double-stranded stem. Yellow ellipsoid is AGO protein, green ellipsoid is Dicer protein, and purple ellipsoid is other proteins such as TRBP and PACT. Black line is mature miR, blue line is PMIS-miR inhibitor.
CONFLICT OF INTEREST

Dr Amendt’s work has been funded by the NIH and Iowa BioVentures Center. He is the CSO of NaturemiR and owns stock in the company, and the new miR inhibitor has been issued a patent. Dr Cao is listed on the patent. All the other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HC conceived hypothesis, performed experiments, analyzed data and wrote the paper; WY performed experiments; XL performed experiments; JW performed experiments; SG performed experiments; NEH performed experiments; TS performed experiments; BAA conceived hypothesis, designed experiments, performed experiments; HC conceived hypothesis, performed experiments, analyzed data and wrote the paper.

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