MicroRNA-directed transcriptional gene silencing in mammalian cells

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MicroRNAs (miRNAs) regulate gene expression at the posttranscriptional level in the cytoplasm, but recent findings suggest additional roles for miRNAs in the nucleus. To address whether miRNAs might transcriptionally silence gene expression, we searched for miRNA target sites proximal to known gene transcription start sites in the human genome. One conserved miRNA, miR-320, is encoded within the promoter region of the cell cycle gene POLR3D in the antisense orientation. We provide evidence of a cis-regulatory role for miR-320 in transcriptional silencing of POLR3D expression. miR-320 directs the association of RNA interference (RNAi) protein Argonaute-1 (AGO1), Polycomb group (PcG) component EZH2, and tri-methyl histone H3 lysine 27 (H3K27me3) with the POLR3D promoter. Our results suggest the existence of an epigenetic mechanism of miRNA-directed transcriptional gene silencing (TGS) in mammalian cells.

miRNA | POLR3D | RNAi | Argonaute | epigenetic

Small noncoding RNAs regulate epigenetic gene silencing pathways that are well-conserved in eukaryotes (1, 2). In particular, conserved components of the RNAi machinery direct the formation of heterochromatin, using sequence-specific small interfering RNAs (siRNAs). Exogenous siRNAs with sequence complementarity to promoter regions have been shown to induce TGS in mammalian cells through heterochromatin assembly (3–6), suggesting that endogenous, small RNA-directed epigenetic processes might exist in mammals. However, endogenous siRNAs or other classes of small RNAs that mediate TGS have yet to be characterized in mammals. miRNAs are endogenous small RNAs that are processed from primary transcripts in the nucleus that have a characteristic stem-loop structure (7). Their biogenesis requires the nuclear RNase III enzyme Drosha, which distinguishes miRNAs from siRNAs and other classes of small RNAs. Although miRNAs are known to mediate posttranscriptional gene silencing (PTGS) in the cytoplasm, recent evidence indicates that at least some fraction of mammalian miRNAs may also have nuclear roles in regulating gene expression (8–10), possibly at the level of transcription.

Results and Discussion

To assess whether endogenous miRNAs might direct TGS in mammals, we performed a bioinformatic search for potential miRNA target sites with perfect sequence complementarity in the promoter regions of well-annotated University of California, Santa Cruz (UCSC) known human genes (11). We analyzed the sequences of promoter regions –1 to –200 bp upstream of known transcription start sites (TSS), which contribute to core promoter activity (12). Within this confined segment of the human genome, we found 10 perfectly matched, potential miRNA target sites (Table 1). Upon further examination, we observed that the primary transcripts containing the stem-loop structures of all 10 miRNAs were encoded within the promoter regions of their proximal known genes, suggesting that these miRNAs could potentially target their opposite strand sequences *in cis* with perfect complementarity.

We proceeded to test this hypothesis by analyzing miR-320, whose mature sequence has been cloned and is conserved in human, mouse, rat, and cow (13). Furthermore, in each of these species, the mature miRNA sequence is located within –1 to –200 bp of the proximal known gene TSS, suggesting the functional importance of this conserved genomic context in the antisense orientation (14) (Fig. 1A). miR-320 is encoded directly upstream of the cell cycle gene POLR3D, which is a conserved subunit specific to RNA polymerase III (15). Because miRNAs are known to exhibit tissue-specific expression, we compared the expression profiles of miR-320 and POLR3D in human brain and testis. Mature miR-320 was highly expressed in brain compared with testis, whereas the inverse was observed for POLR3D mRNA, suggesting a tissue-specific role for miR-320 in regulating POLR3D expression (Fig. 1B).

To specifically assess the contribution of miR-320 to the observed correlation in POLR3D expression, we screened several mammalian cell lines for differential expression of miR-320 and POLR3D. In bEnd.3 mouse cortex cells (16), HeLa human cervical carcinoma cells, and HEK-293 human embryonic kidney fibroblast cells, there was also an inverse correlation between miR-320 and POLR3D abundance (Fig. 1C). Because POLR3D was expressed at relatively low levels in bEnd.3 and HeLa, we transfected these cells with single-stranded control or anti-miR-320 oligonucleotides with perfect complementarity to miR-320 to inhibit its function. Addition of anti-miR-320 substantially decreased the detectable levels of mature miR-320 in both cell types (Fig. 1D). Loss of miR-320 caused a subsequent increase in POLR3D mRNA levels, suggesting a conserved regulatory role for miR-320 in mouse and human cells. We then performed a bioinformatic search for miR-320 seed site matches in the 3' UTRs of mouse and human POLR3D mRNAs. Target prediction algorithms did not reveal the existence of both conserved and poorly conserved seed sites for miR-320 in mouse and human POLR3D 3' UTRs (17), suggesting that miR-320 was not regulating POLR3D expression through a canonical, PTGS-like mechanism. However, a search for miR-320 seed sites in human mRNA 3' UTRs revealed ≈780 predicted target sites (17) (data not shown), some of which may potentially be regulated by miR-320 through PTGS. These results leave open the possibility that miR-320 is also regulating another gene that may potentially affect POLR3D expression indirectly.

Thus, we proceeded to examine whether miR-320 was inducing transcriptional silencing directly at the POLR3D promoter.

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The authors declare no conflict of interest.

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Table 1. MicroRNAs encoded within promoter regions

Chr	Genomic region	Gene	miRNA	Orientation
22	44830062–44830261	AK127117	hsa-let-7b	sense
8	22158364–22158563	NM_001722	hsa-mir-320	antisense
X	133406174–133406373	BC007360	hsa-mir-424	sense
16	15644511–15644710	NM_014647	hsa-mir-484	antisense
3	45705482–45705681	CR749564	hsa-mir-565	antisense
9	122953263–122953462	BC034918	hsa-mir-600	sense
11	61316526–61316725	NM_004111	hsa-mir-611	antisense
15	53452443–53452642	BC034914	hsa-mir-628	sense
17	27701120–27701319	CR616570	hsa-mir-632	sense
19	14501297–14501496	NM_004868	hsa-mir-639	sense

Ten mature miRNAs exhibit perfect sequence complementarity with promoter regions –1 to –200 bp upstream of UCSC known human gene TSS and are transcribed from within these genomic regions on the indicated chromosomes (Chr). Gene accession numbers refer to the downstream genes located proximal to miRNA sequences, and orientation refers to whether the gene and miRNA are transcribed from the same (sense) or opposite (antisense) DNA strands.

Because HEK-293 cells expressed relatively high levels of POLR3D (Fig. 1C), we tested whether POLR3D expression could be attenuated by transfecting cells with mature miR-320 duplexes, compared with cells transfected with control duplexes. Increasing the levels of mature miR-320 in HEK-293 cells resulted in the knockdown of POLR3D mRNA expression, which was assessed at 40 h after transfection (Fig. 2A). Endogenous levels of control miRNAs miR-21 and miR-29b were not affected by introducing additional miR-320 into HEK-293 cells (Fig. 2B). Nuclear run-on experiments (18) in HEK-293 cells, performed at the 20- and 40-h time points after transfection of control or miR-320 duplexes, indicated that increased levels of mature miR-320 induced TGS of the POLR3D gene (Fig. 2C). ChIP assays of HEK-293 cells transfected with control or miR-320 duplexes revealed a miR-320-dependent enrichment of AGO1 at the POLR3D promoter (Fig. 2D), suggesting that miR-320 was directing AGO1 in a sequence-specific manner to its complementary target site within the POLR3D promoter.

Additionally, these results suggested that AGO1 was acting as the effector protein for initiating TGS, analogous to the observed role for AGO1 and other Argonaute proteins in siRNA-mediated TGS in human (4, 19) and other eukaryotic cells (1, 2). Furthermore, H3K27me3 and EZH2, a histone methyltransferase that mediates H3K27me3 (20), were also enriched at the POLR3D promoter in HEK-293 cells with increased levels of miR-320 (Fig. 2D), suggesting an epigenetic role for miR-320 in the regulation of POLR3D expression.

In quiescent fibroblasts, transcriptional silencing of POLR3D expression occurs under serum starvation conditions (15). To examine a potential role for miR-320, HEK-293 fibroblasts were grown for 60 h in the presence of serum or under serum starvation conditions to induce quiescence. POLR3D expression levels decreased upon cell cycle arrest, whereas the expression of mature miR-320 was up-regulated (Fig. 3). To determine whether miR-320 was contributing directly to the silencing of POLR3D expression in the quiescent state, we transfected

Fig. 1. miR-320 levels correlate inversely with POLR3D expression. (A) miR-320 is transcribed from the antisense strand of the POLR3D promoter region and is conserved in human, mouse, rat, and cow. (B–D) Mature miR-320 and POLR3D mRNA expression in human testis and brain tissues (B); bEnd.3, HeLa, and HEK-293 cells (C); and bEnd.3 and HeLa cells transfected with control or anti-miR-320 oligonucleotides (D), as measured by TaqMan miRNA assays and qRT-PCR, respectively, and normalized to GAPDH levels. Error bars indicate SD ($n = 3$).

Fig. 2. miR-320 directs transcriptional silencing of POLR3D. (A) Mature miR-320 and POLR3D mRNA levels in HEK-293 cells transfected with control or miR-320 duplexes, as measured by TaqMan miRNA assays and qRT-PCR, respectively, and normalized to GAPDH levels (relative expression for miR-320 is indicated in hundreds). (B) Mature miR-21 and miR-29b miRNA levels in HEK-293 cells transfected with control or miR-320 duplexes, as measured by TaqMan miRNA assays and normalized to GAPDH levels. (C) Nuclear run-on experiments for nascent POLR3D mRNA transcription in HEK-293 cells transfected with control or miR-320 duplexes, as measured by qRT-PCR and normalized to nascent GAPDH mRNA transcription levels. (D) ChIP assays, using antibodies to AGO1, EZH2, or H3K27me3 or no antibody (NA) controls, in HEK-293 cells transfected with control or miR-320 duplexes. Quantification of immunoprecipitated APRT (control) and POLR3D promoter regions determined by real-time PCR and normalized to input DNA. Error bars indicate SD ($n = 3$).

HEK-293 cells with control or anti-miR-320 oligonucleotides, upon which they were subjected to serum starvation for 60 h. Cells transfected with anti-miR-320 expressed lower amounts of miR-320, whereas POLR3D expression was increased in the quiescent state, suggesting that miR-320 might directly regulate transcriptional silencing of POLR3D expression upon cell cycle arrest (Fig. 3).

Fig. 3. miR-320 regulates POLR3D upon cell cycle arrest. Mature miR-320 and POLR3D mRNA levels in HEK-293 cells grown for 60 h in the presence of serum or under serum starvation conditions, transfected with control or anti-miR-320 oligonucleotides, as measured by TaqMan miRNA assays and qRT-PCR, respectively, and normalized to GAPDH levels. Error bars indicate SD ($n = 3$).

We also detected low levels of sense transcription across the POLR3D promoter, using strand-specific quantitative RT-PCR (qRT-PCR), and the abundance of these promoter transcripts remained relatively constant in both asynchronous and quiescent HEK-293 cell populations, as did the levels of primary miR-320 transcripts (Fig. 4), suggesting that mature miR-320 levels were

Fig. 4. POLR3D promoter RNA transcript expression. Total RNA was prepared from HEK-293 cells grown for 60 h with serum (Asynchronous) or under serum starvation conditions (Quiescent), and levels of primary miR-320 and POLR3D promoter RNA transcript expression were measured using strand-specific qRT-PCR and normalized to GAPDH mRNA levels. Error bars indicate SD ($n = 3$).

Fig. 5. Potential *trans* target genes of miRNAs complementary to promoters. (A) Potential genes with miRNA target sites -1 to -200 bp upstream of UCSC known human gene TSS. Putative miRNA target sites were searched from the sense and antisense strand sequences of gene promoter regions. “mm” refers to the number of mismatches between mature miRNA and target promoter sequences. (B) Most abundantly represented “biological process” gene ontology (GO) categories of annotated genes with putative miRNA target sites containing between 0 and 3 mismatches in gene promoter regions.

regulated posttranscriptionally. The expression levels of the POLR3D promoter RNAs were ≈ 2 orders of magnitude less than those of the primary miR-320 transcripts in the antisense orientation. Sense-stranded promoter RNAs have been implicated in the mechanism of siRNA-mediated TGS in human cells (3), although we have not investigated a requirement for sense transcription across the promoter in miR-320 regulation of POLR3D. However, sense transcription across the POLR3D promoter, which encodes the miR-320 sequence in the antisense orientation, would provide a target site-containing transcript *in cis* with perfect sequence complementarity.

These data suggest a *cis*-regulatory role in which miR-320 participates in a negative feedback loop at the POLR3D promoter to induce TGS, but miRNAs may potentially function more broadly in transcriptional silencing to target gene promoters *in trans*. We expanded our bioinformatic analyses to allow for progressively less stringent base pairing requirements. Permitting 1–2 mismatches between mature miRNAs and promoter sequences -1 to -200 bp upstream of known human gene TSS identified several candidate target genes. However, permitting up to 3 mismatches identified $\approx 1,200$ candidate target genes in the human genome with near perfect sequence complementarity within core promoter regions (Fig. 5A). The gene ontology (GO) “biological process” categories (21) containing the highest percentage of the $\approx 1,200$ candidate target genes represented genes involved in regulation of transcription or apoptosis (Fig. 5B). We also performed an analysis for enrichment of conserved miRNA seed sequences (22) within core promoter regions, which indicated a small but statistically significant enrichment. Seed sequences from highly conserved miRNAs were enriched $\approx 5\%$, compared with control seed sequences, within human gene promoters ($P = 0.05$, permutation test). Further experiments may reveal the extent to which mammalian miRNAs might potentially regulate transcription *in trans*.

The results presented here suggest a cell cycle-dependent role for a mammalian miRNA in TGS. Although several studies in mammalian cells have now described the observation that exogenous siRNAs targeted to promoter regions can induce heterochromatin formation (3–6), an endogenous pathway for this phenomenon has not been previously described. We provide evidence that the endogenous miRNA pathway may direct transcriptional silencing in the nucleus, in addition to the cytoplasmic PTGS and translational activation (23) pathways. Exogenous siRNAs have been shown to direct TGS of cancer and HIV-related genes in mammalian cells (24), and misregulation of endogenous miRNAs that target gene promoters may potentially play a role in the aberrant epigenetic silencing of cancer-related genes, including genes that regulate apoptosis or Ras signaling (Fig. 5B). Additionally, recent findings in *Drosophila* and *C. elegans* point to a connection between RNAi, miRNAs, and PcG silencing (25, 26). Taken together with the results described here, this suggests a potentially conserved role for the miRNA pathway in transcriptional and epigenetic gene silencing.

Materials and Methods

Mapping MicroRNAs to Gene Promoter Regions. We mapped the occurrences of potential miRNA target sites within promoter regions, using the UCSC table browser to download -1 to -200 bp upstream of the annotated TSS of UCSC known human genes (UCSC March 2006, National Center for Biotechnology Information Build 36.1). We downloaded mature human miRNA sequences from miRBase and searched for occurrences of potential miRNA binding sites in the sense and antisense strands of promoter sequences. Ten miRNAs have perfect sequence complementarity within the promoter sequences and are transcribed from these promoter regions. Decreasing the stringency for sequence complementarity by allowing 1, 2, or 3 mismatches between miRNAs and promoter regions yielded *trans* candidate target genes.

RNA Samples and Cell Culture. FirstChoice Total RNA from human brain and testis (Ambion) or total RNA isolated from bEnd.3 (ATCC), HeLa (ATCC), and HEK-293 (ATCC) cells, using RNA STAT-60 Reagent (Tel-Test), according to manufacturer recommended protocols, were used for TaqMan miRNA assays

and qRT-PCR. bEnd.3 and HeLa cells were transfected with control or anti-miR-320 oligonucleotides (Ambion) at 50 nM final concentration, using TransIT-siQUEST transfection reagent (Mirus). HEK-293 cells were transfected with control or miR-320 duplexes (Ambion) and with control or anti-miR-320 oligonucleotides with proprietary chemical modifications [Ambion; product description for anti-miR-320 [AM11621], which exclusively targets the mature miR-320 sequence with perfect complementarity; from www.ambion.com: "Anti-miR miRNA Inhibitors are chemically modified, single stranded nucleic acids designed to specifically bind to and inhibit endogenous microRNA (miRNA) molecules"] at 50 nM final concentration, using Lipofectamine 2000 (Invitrogen), according to manufacturer recommended protocols, and total RNA for each cell line was isolated after transfections in 6-well plates, plated 24 h before transfections. For cell cycle analysis, HEK-293 cells were grown for 60 h with serum or under serum starvation conditions to induce quiescence, which was confirmed by FACS analysis.

TaqMan MicroRNA Assays. Mature miRNA quantification was performed using TaqMan MicroRNA Assays for miR-320, miR-21, and miR-29b, according to manufacturer recommended protocols (Applied Biosystems). Ten nanograms of total RNA, 50 nM stem-loop RT primer, RT buffer, 0.25 mM each dNTP, 3.33 units/ml MultiScribe reverse transcriptase (RT), and 0.25 units/ml RNase inhibitor were used in 15- μ L RT reactions for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C, using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems). For real-time PCR, 1.33 μ L (1:15 dilution) of cDNA, 0.2 mM TaqMan probe, 1.5 mM forward primer, 0.7 mM reverse primer, and TaqMan Universal PCR Master Mix (Applied Biosystems) were added in 20- μ L reactions for 10 min at 95 °C and 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. All real-time PCR experiments were done using an iCycler iQ system (Bio-Rad).

Quantitative RT-PCR. The SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) was used to amplify POLR3D and GAPDH (normalization control for qRT-PCR and TaqMan MicroRNA Assays) mRNAs from total RNA samples, according to manufacturer recommended protocols. For strand-specific qRT-PCR, SuperScript III RT (Invitrogen) was used to generate cDNA, according to manufacturer recommended protocols, and iQ SYBR Green Supermix (Bio-Rad) was used to perform real-time PCR. The following primers were used for qRT-PCR and qPCR: hPOLR3D mRNA Fw AGAAGGAGAAGCGT-GAAAGGGACA, hPOLR3D mRNA Rv ATCGTCCTTCTCCAGCATACGCAA, hGAPDH mRNA Fw CGCTCTCTGCTCTCTCTGT, hGAPDH mRNA Rv CCATGGT-GTCTGAGCGATGT, mPolr3d mRNA Fw ATGCTTGACCTTGGCTGACCTGA, mPolr3d mRNA Rv TCTGTCCATCTCACCGGTGTTTGT, mGapdh mRNA Fw TTCACCACTGGAGAAGGC, mGapdh mRNA Rv CCCTTTGGCTCCACCTT, hPOLR3D prom-1 Fw CAGACCACTCACCTCATCTTT, hPOLR3D prom-1 Rv AG-TATTTATCAGGCGGCGCTTC, hPOLR3D prom-2 Fw ATTCCTCCAGGCTTTGAC-CAAGA, hPOLR3D prom-2 Rv CATGGTGACCTTGGAGTGGTCATT, hAPRT prom Fw GCCTTGACTCGCACTTTTGT, and hAPRT prom Rv TAGGCGCCATCGATTTAAG.

Nuclear Run-On. Nuclear run-on assays were performed as described in ref. 18. HEK-293 cells were transfected with control or miR-320 duplexes (Ambion) at 50 nM final concentration, using Lipofectamine 2000 (Invitrogen). Twenty and 40 h after transfection, 2×10^7 cells were washed with cold PBS, harvested, and lysed on ice in 0.5% Nonidet P-40 lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl₂], and centrifuged at $500 \times g$ for 10 min. Supernatants were removed and nuclei were incubated in reaction buffer [10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.3 mM KCl] and 2.5 mM NTP plus Biotin-16-UTP mix (Roche) for 45 min at 30 °C. The transcription reaction was stopped by adding RNA STAT-60 Reagent (Tel-Test) to isolate total nuclear RNA, according to manufacturer recommended protocols. Biotinylated nascent RNA transcripts were isolated by incubation with streptavidin beads (Active Motif) for 2 h at room temperature on a rocking platform. Beads were collected by centrifugation

and washed once with $2 \times$ SSC-15% formamide for 10 min and twice with $2 \times$ SSC for 5 min on a rocking platform. Biotinylated RNA was eluted from streptavidin beads in H₂O or 10 mM EDTA pH 8.2 by incubation at 90 °C for 10 min and analyzed by qRT-PCR.

Chromatin Immunoprecipitation. HEK-293 cells were transfected with control or miR-320 duplexes (Ambion) at 50 nM final concentration, using Lipofectamine 2000 (Invitrogen). Cells (2×10^7) were cross-linked with 1% formaldehyde for 10 min at room temperature. Using the EZ-ChIP Kit (Upstate Biotechnology), $10 \times$ Glycine was added to stop cross-linking, and cells were washed with cold PBS and harvested in SDS lysis buffer plus Protease Inhibitor Mixture II (Upstate Biotechnology). Cell lysates were sonicated using a Bioruptor sonicator (Diagenode) with refrigerated water bath and a rotating 1.5-mL microtube unit for 5 cycles of 30 sec ON and 30 sec OFF at HIGH setting, generating ≈ 500 -bp DNA fragments according to manufacturer recommended protocols. Extracts were precleared with 60 μ L of 50% Protein G Agarose slurry (Upstate Biotechnology) for 1 h at 4 °C with rotation. Immunoprecipitations were performed using 5 μ g each of Anti-AGO1 (Upstate Biotechnology; catalog no. 07-599), Anti-EZH2 (Upstate Biotechnology; catalog no. 07-400), and Anti-H3K27me3 (Upstate Biotechnology; catalog no. 07-449) overnight at 4 °C with rotation, along with no antibody controls. Sixty microliters of 50% Protein G Agarose slurry (Upstate Biotechnology) was added to each sample for 1 h at 4 °C with rotation to recover the antibody/antigen/DNA complexes, which were washed 5 times, using buffers from the EZ-ChIP Kit (Upstate Biotechnology) as recommended. Eluted samples were incubated overnight at 65 °C to reverse cross-links and treated with RNase A (Upstate Biotechnology) at 37 °C for 30 min and Proteinase K (Upstate Biotechnology) at 45 °C for 1 h. DNA purification was performed using spin columns (Upstate Biotechnology), and iQ SYBR Green Supermix (Bio-Rad) was used to perform real-time PCR on an iCycler iQ system (Bio-Rad) with promoter-specific primers.

Gene Ontology Classification of Candidate microRNA Target Genes. Gene ontology (GO) analysis was performed using Fatigo (21). The set of $\approx 1,200$ genes with putative miRNA target sequences upstream of known gene TSS was used as input data for Fatigo analysis under the "Biological Process" (level 8) GO category. The 7 most highly represented "Biological Process" categories are shown in Fig. 5B. All other categories each represented 3% or less of the input set of genes.

MicroRNA Seed Enrichment. A set of 7-mer seed sequences from highly conserved miRNAs were shuffled to obtain control seeds as described in ref. 22, except that we used the core promoter sequences –1 to –200 bp upstream of known human gene TSS instead of 3' UTRs to generate the controls. For each miRNA seed, we counted the number of occurrences of highly conserved seed sites in the core promoter sequences, as defined by the UCSC 17-Way Most Cons track (UCSC April 2006, National Center for Biotechnology Information Build 36.1) but excluding the miRNA sequences mapping to the promoter region. We then repeatedly ($n = 1,000$) compared the total number of such seed sites with the same count for an equal number of randomly selected control seeds. The number of times that the seed occurrence counts for the real miRNAs were less than or equal to the seed occurrence counts for the random control sets estimated the P value of miRNA seed enrichment. The total number of miRNA seed sites in promoter regions was 3,681, compared with $3,490 \pm 121.8$ control seed sites (5.5% enrichment; and $P = 0.053$).

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