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miR-24 Inhibits Cell Proliferation by Targeting E2F2, MYC, and Other Cell-Cycle Genes via Binding to "Seedless" 3′UTR MicroRNA Recognition Elements

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SUMMARY

miR-24, upregulated during terminal differentiation of multiple lineages, inhibits cell-cycle progression. Antagonizing miR-24 restores postmitotic cell proliferation and enhances fibroblast proliferation, whereas overexpressing miR-24 increases the G1 compartment. The 248 mRNAs downregulated upon miR-24 overexpression are highly enriched for DNA repair and cell-cycle regulatory genes that form a direct interaction network with prominent nodes at genes that enhance (*MYC*, *E2F2*, *CCNB1*, and *CDC2*) or inhibit (*p27Kip1* and *VHL*) cell-cycle progression. miR-24 directly regulates *MYC* and *E2F2* and some genes that they transactivate. Enhanced proliferation from antagonizing miR-24 is abrogated by knocking down *E2F2*, but not *MYC*, and cell proliferation, inhibited by miR-24 overexpression, is rescued by miR-24-insensitive *E2F2*. Therefore, *E2F2* is a critical miR-24 target. The *E2F2* 3′UTR lacks a predicted miR-24 recognition element. In fact, miR-24 regulates expression of *E2F2*, *MYC*, *AURKB*, *CCNA2*, *CDC2*, *CDK4*, and *FEN1* by recognizing seedless but highly complementary sequences.

INTRODUCTION

MicroRNAs (miRNAs) regulate key steps of cell differentiation and development by suppressing gene expression in a sequence-specific manner (Bartel, 2009). In mammals, the active strand miRNA sequence (typically ~22 base pairs) is partially complementary to binding sites in the 3′UTR of genes, often with full complementarity to 7 or 8 nucleotides in the "seed region" (residues 2–9) of the miRNA. Gene suppression in mammals is thought to occur primarily by inhibiting translation (Olsen and Ambros, 1999). However, miRNAs in mammals also cause mRNA decay (Chang et al., 2007; Lim et al., 2005; Johnson et al., 2007); recent reports (Baek et al., 2008; Selbach et al., 2008) suggest that reduced protein is frequently associated with decreased mRNA.

miR-24 is consistently upregulated during terminal differentiation of hematopoietic cell lines into a variety of lineages (Lal et al., 2009). miR-24 is also upregulated during thymic development to naive CD8 T cells (Neilson et al., 2007) and during muscle and neuronal cell differentiation (Sun et al., 2008; Fukuda et al., 2005). miR-24 is encoded with miR-23 and miR-27 in two duplicated gene clusters. One cluster (miR-23b, miR-27b, and miR-24-1) is within a chromosome 9 EST, and the other (miR-23a, miR-27a, and miR-24-2) is in a chromosome 19 intergenic region. Both miR-24 genes are processed to the same active strand. Disruption or changes in expression of both sites have been linked to CLL prognosis (Calin et al., 2005). Because miR-24 is upregulated in diverse cell types during terminal differentiation, we sought to identify its function and the target genes that it regulates.

Common approaches to identify miRNA target genes are (1) bioinformatic algorithms that predict potential target genes that contain conserved 3′UTR sequences complementary to a seed region at the 5′ end of the miRNA active strand (Doench and Sharp, 2004; Lewis et al., 2005), (2) analysis of mRNAs that are downregulated when a miRNA is overexpressed (Chang et al., 2007; Johnson et al., 2007; Lim et al., 2005), and (3) identifying mRNAs enriched in coimmunoprecipitates with tagged Argonaute or GW182 proteins in cells overexpressing the miRNA (Easow et al., 2007; Zhang et al., 2007). The bioinformatic approach is hampered by the fact that the existing algorithms have a high margin of error (most predicted genes are not real targets, and some key targets, *such as RAS* for let-7, are not predicted [Johnson et al., 2005]). The utility of the biochemical approach involving Argonaute proteins for genome-wide target

identification of miRNAs is still unclear because Argonaute overexpression globally increases miRNA levels, perhaps obscuring the effect of an individual overexpressed miRNA (Diederichs and Haber, 2007). Because miRNA-mediated mRNA degradation and protein downregulation often occur together (Baek et al., 2008), identifying the mRNAs that decrease when a miRNA is overexpressed might identify many of its targets. Although some bona fide miR-24 targets that are primarily regulated by translation will be missed by this approach and other downregulated genes may not be directly regulated, this strategy has been successfully used to identify targets of some mammalian miRNAs, including miR-124 and miR-1 (Lim et al., 2005), miR-34a (Chang et al., 2007), and let-7 (Johnson et al., 2007). Therefore, we applied this approach to identify the genes regulated by miR-24 in HepG2 cells that express low levels of miR-24 and combined it with bioinformatics to uncover miR-24-regulated pathways. We find that miR-24 regulates a network of genes that control cell-cycle progression and DNA repair (Lal et al., 2009). Overexpressing miR-24 increases the G1 population and reduces DNA replication, whereas antagonizing miR-24 increases cell proliferation, which can be rescued by knocking down *E2F2*, suggesting that *E2F2* is a key miR-24 target gene. *MYC* and other genes important in cell-cycle regulation that are transcriptionally regulated by MYC and E2Fs (*AURKB*, *BRCA1*, *CCNA2*, *CDC2*, *CDK4*, and *FEN1*) are also direct miR-24 targets by luciferase assay. Of note, *E2F2* and most of these genes lack 3′UTR miR-24 seed match sequences. However, miR-24 regulates these genes by base pairing to "seedless" 3′UTR MREs with extensive base pairing elsewhere in the sequence.

RESULTS

miR-24 Is Upregulated during Hematopoietic Differentiation

To understand the role of miRNAs during terminal differentiation, we analyzed miRNA expression by microarray in two human leukemia cell lines: K562 cells differentiated to megakaryocytes using 12-O-tetradecanoylphorbol-13-acetate (TPA) or to erythrocytes with hemin and HL60 cells differentiated to macrophages using TPA or to monocytes using vitamin D3. miR-24 was one of only six miRNAs that was consistently upregulated in all four systems of terminal differentiation (Lal et al., 2009). The other uniformly upregulated miRNAs were three other members of the miR-24 clusters (miR-23a, miR-23b, and miR-27a), miR-22, and miR-125a. miR-24 was the most upregulated of these miRNAs. We therefore focused on miR-24, which we hypothesized might regulate terminal differentiation in multiple cell lineages. qRT-PCR confirmed the induction of miR-24 during differentiation of these hematopoietic cells (Figure 1A) with the highest upregulation in K562 cells treated with TPA. The mature miR-24 transcript increased 2- to 8-fold during differentiation into megakaryocytes, erythrocytes, macrophages, monocytes, and granulocytes. Expression of the chromosome 19 miR-24 cluster primary transcript encoding miR-23a, miR-27a, and miR-24 increased in both cell lines within 6 hr of TPA treatment, peaked at ~12 hr, and remained elevated for at least 2 days (Figures S1A and S1B available online), suggesting that the observed increase in mature miR-24 was due to increased transcription. Upregulation of the Dicer-cleaved mature miRNA was slightly delayed, becoming significant at 12–16 hr (Figures S1C and S1D). Mature miR-24 levels remained elevated for as long as was measured (4 days).

miR-24 Inhibits Cellular Proliferation by Increasing the G1 Compartment

Because cessation of cell proliferation is a hallmark of terminal differentiation, we first examined whether proliferation is altered by either inhibiting or enhancing miR-24 function by transfecting cells with miR-24 2′-OMe antisense oligonucleotide (ASO) or miRNA mimics, respectively. When K562 cells were transfected with miR-24 ASO, miR-24 was dramatically and specifically reduced by qRT-PCR 36 hr later (Figure 1B). DNA replication, measured by thymidine incorporation, doubled in cells transfected with miR-24 ASO compared to cells transfected with control ASO (Figure 1C). When K562 cells were differentiated with TPA for 4 hr, thymidine incorporation declined by 60%. However, in cells transfected with miR-24 ASO and treated with TPA, thymidine uptake was indistinguishable from that of the control ASO-transfected, but TPA-untreated, cells (Figure 1C). Therefore, miR-24 ASO fully restored proliferation to differentiating K562 cells. To examine whether miR-24 also inhibits cell proliferation in nontransformed cells, we next antagonized miR-24 in early passage WI-38 and IMR-90 normal diploid fibroblasts. Antagonizing miR-24 in WI-38 and IMR-90 cells dramatically reduced miR-24 (Figure 1D) and increased thymidine uptake > 2-fold 48 hr after transfection (Figure 1E). Conversely, overexpressing miR-24 in HepG2 cells synchronized with nocodazole, which typically leads to mitotic arrest and only ~8% of cells in G1, increased G1 cells 3-fold (22%; miR-24 versus cel-miR-67, $p < 0.001$) (Figure 1F).

We next analyzed how miR-24 expression changes during normal cell-cycle progression using K562 cells released at various times from nocodazole treatment, which synchronized them in G2/M (Bar-Joseph et al., 2008; O'Donnell et al., 2005) (Figures 1G and 1H). Before release, 90% of cells were in G2/M; 8 hr later, 65% were in G1; and 12 hr after removing nocodazole, 45% were in S phase. miR-24 was low in G2/M, increased > 3-fold by 8 hr when most cells were in G1, and then declined by 12 hr as cells progressed into S phase. These results suggest that miR-24 is most highly expressed in G1. Taken together with our finding that cells transduced with miR-24 mimics accumulate in G1, these results suggest that miR-24 regulates cell-cycle progression mostly by blocking or delaying the G1/S transition.

Most Genes Downregulated by miR-24 Contain a miR-24 Seed in Their 3′UTR

We next sought to identify miR-24-regulated targets and cellular pathways by comparing mRNA microarrays of cells transfected with miR-24 or control miRNA (cel-miR-67) mimic. Transfecting HepG2 cells, which have low endogenous miR-24 levels (Figure 2A), with a miR-24 mimic increased miR-24 expression ~80-fold compared with control cells (Figure 2B). Total RNA, isolated from duplicate miRNA-transfected samples 48 hr later, was amplified, labeled, and hybridized to Illumina mRNA microarrays. 248 mRNAs were downregulated at least 2-fold by miR-24 overexpression (Z ratio > 1.5) (Table S1). We validated the microarray

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