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WEE1 is a validated target of the microRNA *miR-17-92* cluster in leukemia

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Abstract

MicroRNAs are short single-stranded RNAs that regulate target gene expression by binding to complementary sites in the 3' untranslated region of their mRNA targets. The polycistronic *miR-17-92* cluster, which encodes *miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92a*, was previously shown to be overexpressed in multiple types of cancer. In the present study, target gene prediction algorithms were used to predict potential targets of the *miR-17-92* cluster. WEE1, a kinase that inhibits cell cycle progression, was identified as a possible target of five of the six miRNAs of the cluster. Luciferase reporter assays were used to determine that *miR-17*, *miR-20a*, and *miR-18a* specifically target nucleotides 465 to 487 of the 3' UTR of *WEE1*, while *miR-19a* and *miR-19b* exert control on *WEE1* by targeting nucleotides 1069 to 1091. A negative correlation was determined between endogenous *miR-17* or *miR-19a* expression and endogenous WEE1 protein expression in the same panel of cell lines. We conclude that WEE1 is a valid target of the *miR-17-92* cluster in leukemia.

Keywords

MicroRNA; *miR-17-92*; WEE1; leukemia

Introduction

MicroRNAs (miRNAs, miRs) are approximately 22 nucleotide noncoding RNAs that function as antisense regulators of messenger RNAs (mRNAs) (1). miRNAs utilize different mechanisms to downregulate gene expression of their targets. When there is a high degree of

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complementarity to the mRNA sequence, the target mRNA is cleaved within the miRNA-binding site, which results in decreased mRNA and protein levels (2). However, when there is a lower degree of complementarity to the mRNA sequence, translation is repressed, which results in decreased protein levels (2).

Although many miRNAs are encoded by individual genes, some are located in clusters containing multiple miRNAs. MiRNA clusters are expressed together as a long precursor RNA which is then processed into individual miRNAs (3). The *miR-17-92* cluster is located within an 800 base-pair region of chromosome 13 (4). This cluster encodes six miRNAs (*miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92a*) (5). Several of the miRNAs of the *miR-17-92* cluster share sequence homology that extends beyond the seed targeting region, which suggests that they might be able to act collectively on some common targets. The *miR-17-92* cluster is overexpressed in B-cell lymphoma, where it acts as an oncogene (6). Overexpression of *miR-17-92* accelerates Myc-dependent B-cell lymphoma development (7, 8). The Chen group used bead-based miRNA expression profiling assays and TaqMan qPCR assays to show that the individual miRNAs of the *miR-17-92* cluster are specifically upregulated in *MLL* rearranged leukemias, but not in the other subtypes that they tested (9, 10). These miRs are also overexpressed in solid tumors including those originating from the breast, colon, lung, pancreas, prostate, and stomach (11).

WEE1 is a protein kinase that adds an inhibitory phosphate on Tyr15 of cyclin dependent kinase 1 (Cdk1) during interphase (12). WEE1 holds Cdk1 in an inactive state until the G2/M transition of the cell cycle. The function of WEE1 is antagonized by Cdc25 phosphatase, which removes the inhibitory phosphate at the onset of the M phase (13). Here, we identified WEE1 as a putative target of the *miR-17-92* cluster and set out to validate this novel regulatory relationship in the context of leukemia.

Materials and methods

MicroRNA Target Gene Prediction

Prediction algorithms including TargetScan (www.targetscan.org), MicroCosm Targets (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5), PicTar (pictar.mdc-berlin.de), and miBridge (sitemaker.umich.edu/mibridge/target_predictions) were used to predict potential biological targets of the *miR-17-92* cluster.

Cloning of Luciferase Reporter Constructs

The 3' UTR of *WEE1* (nucleotides 2418 – 3356 of NM_003390.3) was amplified from human genomic DNA using forward primer (5'ATGTTACACCAGCCTTTCCAGGGT3') and reverse primer (5'AGACAATTAAGGTAAGCTCAGAGTGA3'). To add flanking restriction sites to the ends of the PCR product to facilitate cloning into the reporter vector, the following forward primer (5'TCTCTCTCTACTAGTATGTTACACCAGCCTTTCCAGGGT3') and reverse primer (5'TCTCTCTCTAAGCTTAGACAATTAAGGTAAGCTCAGAGTGA3') were used. The 3' UTR of *WEE1* and the pMIR-REPORT Luciferase vector (Applied Biosystems, Catalog #AM5795) were digested with SpeI and HindIII, ligated and electroporated into DH10β

cells to create a construct with the 3' UTR of *WEE1* immediately following the luciferase coding sequence. A construct with the putative *miR-17*, *miR-20a*, and *miR-18a* target site mutated (referred to as miR-17, 20a, 18a Mut) was produced using a modified site-directed mutagenesis protocol with non-overlapping primers (forward primer: 5'GACTTGATATATCCCACTGGGAGACAGGGGTAGGCATTGCATGAACCATGGGATG3'; and reverse primer: 5'GCCAATCAATGTTAATAAAACACAAGTCAAAGACAATGTACCACATGTTTTAGACC3') on the wild-type luciferase reporter template. The mutated region was ligated into the pMIR-REPORT Luciferase vector using the SpeI and HindIII restriction sites used previously. To generate the construct with the putative *miR-19a* and *miR-19b* target site mutated (referred to as miR-19a, 19b Mut) and the construct with all the putative *miR-17-92* target sites mutated (referred to as All Mut), the forward primer (5'TCTCTCTCTACTAGTATGTTACACCAGCCTTTCCAGGGT3') and reverse primer (5'CCTTTATTAAGCTTAGACAATTAAGGTAAGCTCAGAGTGACTTTTAATATGCCAATCAATGTTAATAAAACACAAGTCAAAGACAATGTACCACATGTTTTAGACC3') were used on the wild type and miR-17, 20a, 18a Mut plasmids, respectively. All plasmids were confirmed by sequencing.

Luciferase Reporter Assays

Sixty thousand HEK293T cells were plated in 24-well plates and transfected after 24 hours using 6 ng pRL-TK control vector (Promega, Catalog #e2241), 120 ng Luciferase *WEE1* 3' UTR (Wild-type, miR-17, 20a, 18a Mut, miR-19a, 19b Mut, or All Mut), and 600 ng MSCV-PIG plasmid (Empty Vector, miR-17, miR-17-19b, or miR-17-92, provided by J. Chen, University of Chicago (10)). The reporter assay was performed 42 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega, Catalog #E1910) according to the manufacturer's protocol. Firefly luciferase and *Renilla* luciferase were measured using a microplate luminometer from Veritas. The data was analyzed by determining the relative luciferase (firefly luciferase: *Renilla* luciferase) and normalizing to the wild-type luciferase reporter. The experiment was performed in triplicate and repeated three times.

Cell Culture

MV-4-11, K-562, and HL-60 cells were cultured in IMDM (Gibco), 10% FBS, and 1% Pen/Strep. RS4;11, THP-1, MonoMac6, and U-937 cells were cultured in RPMI-1640 (HyClone), 10% FBS, 1% Pen/Strep, and 0.05 mM 2-mercaptoethanol. HEK293T cells were cultured in DMEM (HyClone), 10% FBS, and 1% Pen/Strep. All cells were cultured in a 37°C incubator with 5% carbon dioxide.

RNA Isolation, cDNA Synthesis, and TaqMan MicroRNA Assays

RNA was isolated from MV4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T cells according to the manufacturer's protocol (Sigma, Catalog #T9424). Ten ug of total isolated RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Catalog #4366596) and RT primers specific to either *U6* snRNA (constitutively expressed control), *miR-17*, or *miR-19a*. During the PCR

amplification step, AmpliTaq Gold DNA polymerase was used to amplify target cDNA using sequence-specific primers from the TaqMan MicroRNA Assay Kit (Applied Biosystems). Real-time PCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems), and the data was analyzed with ABI Prism 7300 software. *MiR-17* and *miR-19a* expression levels were determined relative to *U6* snRNA levels using the 2^{-C_t} method (14). Relative expression was arbitrarily normalized to MV-4-11 expression levels. The assay was performed in triplicate and repeated two to five times.

Nuclear and Cytoplasmic Protein Extraction

All steps for nuclear and cytoplasmic protein extraction were performed on ice. One million MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T cells were pelleted and resuspended in 400 μ L cold Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1:100 Protease inhibitor cocktail (Sigma, Catalog #P8340)) by gentle pipetting. The cells were allowed to swell on ice for 15 minutes. Twenty five μ L of 10% Nonidet NP-40 (Calbiochem, Catalog #492015) was added and the tubes were vigorously vortexed for 10 seconds. The tubes were centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatants containing the cytoplasmic extracts were snap frozen and stored at -80°C until further analysis. The nuclear pellet was resuspended in 50 μ L ice-cold Buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1:100 Protease inhibitor cocktail (Sigma, Catalog #P8340)). The tubes were rocked for 15 minutes at 4°C and then centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatants containing the nuclear extracts were snap frozen and stored at -80°C until further analysis.

Western Blot Analysis

Thirty μ L of cytoplasmic or nuclear extracts were electrophoresed on 10% SDS-polyacrylamide gels. Gels were transferred to Immobilon transfer membranes (Millipore, Catalog #IPVH00010). The membranes were blocked in 5% BSA for 1 hour at room temperature and then incubated with 1:1000 WEE1 rabbit polyclonal antibody (Cell Signaling, Catalog #4936) followed by incubation with ECL Anti-Rabbit IgG-HRP secondary antibody (GE Healthcare, Catalog #NA934V). The membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Catalog #34078) and developed using a Fujifilm LAS-3000 Luminescent Image Analyzer with Image Reader LAS-3000 software. To normalize for loading, the membranes were stripped with Mild Stripping Buffer (15 g Glycine, 1 g SDS, 10 mL Tween-20, final volume brought up to 1 L using dH₂O, pH 2.2). The membranes were blocked with 5% BSA as described above and then incubated with 1:5000 β -Actin mouse monoclonal antibody (Sigma, Catalog #A5441) followed by incubation with ECL Anti-Mouse IgG-HRP secondary antibody (GE Healthcare, Catalog #NA931V). Band intensity from western blot images was quantified using MultiGauge V3.0 software (Fujifilm) by subtracting background from band intensity. WEE1 expression was determined relative to β -Actin expression and was arbitrarily normalized to MV4-11 expression levels. The experiment was repeated twice.

Results

***WEE1* is a Predicted Target of Multiple miRs within the *miR-17-92* Cluster**

Bioinformatic miRNA target gene prediction algorithms were used to predict potential targets of *miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92a*. For each miRNA of the cluster, a list was compiled of the thirty target genes with the highest total context score from TargetScan, the twenty target genes with the lowest P-value from MicroCosm Targets, the twenty target genes with the highest PicTar score from PicTar, and all predicted target genes from miBridge. Both MicroCosm Targets and miBridge predicted *WEE1* to be a high probability target of *miR-17* and *miR-20a*. *WEE1* also was identified as a likely target of *miR-18a*, *miR-19a*, and *miR-19b* in one prediction program each.

The putative *miR-17-92* target sites within the 3' UTR of *WEE1* are shown in Figure 1A. Since *miR-17*, *miR-20a*, and *miR-18a* are highly homologous, it is not surprising that they are all predicted to target the same region in the 3' UTR of *WEE1*. Similarly, *miR-19a* and *miR-19b* share a common predicted target site. The predicted binding between *WEE1* mRNA and each of the individual miRNAs of the cluster shows that there is a high degree of sequence complementarity within each pairing especially in the seed region (Figure 1B).

The 3' Untranslated Region of *WEE1* is Specifically Targeted by *miR-17-92*

To establish whether *WEE1* is a biological target of the *miR-17-92* cluster and to determine the specific regions being targeted, luciferase reporter assays were performed. First, the 3' UTR of *WEE1* with the putative *miR-17-92* binding sites intact was amplified from human genomic DNA and cloned into the pMIR-REPORT Luciferase vector immediately following the luciferase coding sequence (Figure 2A). Three mutant versions of the reporter construct were also generated to disrupt binding between the miRNA and the mRNA by specifically mutating the six nucleotides where the seed region of the miRNA is predicted to bind. One mutant possesses mutations in the putative *miR-17*, *miR-20a*, and *miR-18a* target site while another mutant contains mutations in the putative *miR-19a* and *miR-19b* target site (Figure 2A). The third mutant has all the putative *miR-17-92* target sites mutated (Figure 2A).

The wild-type or one of the three mutant reporter constructs were co-transfected with empty vector, *miR-17*, *miR-17-19b*, or *miR-17-92* expression plasmids (10) in HEK293T cells. If *WEE1* is a target of the miRs, when the wild-type reporter construct is co-expressed with any of the relevant miRNAs of the cluster, luciferase expression should be decreased; mutation of the target binding sites would inhibit binding and result in relatively higher luminescence. As seen in Figure 2C, when the putative *miR-17* binding site is mutated in the reporter construct (*miR-17*, *miR-20a*, and *miR-18a* target site mutated or all target sites mutated), relative luciferase activity increases upon overexpression of *miR-17* as compared to when the putative binding site is intact (wild-type or *miR-19a* and *miR-19b* target site mutated).

The *miR-17-19b* construct expresses all of the miRNAs present in the *miR-17-92* cluster except for *miR-92a* which is not predicted to target *WEE1*. When the *miR-17*, *miR-20a*, and *miR-18a* target site is mutated in the reporter construct, overexpression of *miR-17*, *miR-20a*, and *miR-18a* leads to an increase in luciferase activity (Figure 2D). When the *miR-19a* and

miR-19b target site is mutated in the reporter construct, overexpression of *miR-19a* and *miR-19b* causes a small, but statistically significant increase in luciferase activity (Figure 2D). When all of the predicted target sites are mutated, co-expression of all five miRNAs increases luciferase activity by a larger margin (Figure 2D).

As shown in Figure 2E, co-expression of the entire *miR-17-92* cluster with any of the mutant reporter constructs results in a statistically significant increase in luciferase activity. The reporter construct with all of the predicted target sites mutated had the greatest increase in luciferase activity relative to the wild-type construct.

There were small, but statistically significant increases in luciferase activity in the presence of the empty vector (Figure 2B). In support of this result, HEK293T cells express endogenous *miR-17-92* (Figure 3).

We conclude that *miR-17* specifically targets nucleotides 465 to 487 of the 3' UTR of *WEE1*. *miR-20a* and *miR-18a* also potentially regulate *WEE1* by targeting the same region in the 3' UTR, albeit to a lesser extent than *miR-17*. Finally, *miR-19a* and *miR-19b* exert control on *WEE1* by targeting nucleotides 1069 to 1091 of the 3' UTR, although this regulation is likely less influential than that of *miR-17*.

Determination of miR-17-92 Expression Levels in Leukemia Cell Lines

To quantify endogenous *miR-17-92* expression in leukemia cell lines, RNA was isolated, cDNA synthesized using a looped reverse transcription primer specific to each miRNA, and TaqMan MicroRNA Assays were performed using a variety of cell lines. The leukemia cell lines analyzed included those with MLL fusions (MV-4-11, RS4;11, THP-1 and MonoMac6), as well as those caused by different mutations. These non-MLL leukemia cell lines included one with a *BCR-ABL* gene fusion (K-562), one with amplified *c-Myc* (HL-60), and one with a *CALM-AF10* fusion arising from a histocytic lymphoma (U-937) (15). Also included was a non-leukemia cell line (HEK293T).

Two representative miRNAs, *miR-17* and *miR-19a*, were chosen to quantify. *miR-17* was analyzed because *miR-17*, *miR-20a*, and *miR-18a* have highly homologous sequences and are predicted to target the same site in the 3' UTR of *WEE1*. Similarly, because *miR-19a* and *miR-19b* share the same target site, *miR-19a* was selected as the second representative miRNA.

All cell lines showed detectable levels of *miR-17* (Figure 3A) and *miR-19a* (Figure 3B), varying over a 5-fold range. There was no correlation between expression level and type of leukemia cell line, with the highest expression of both miRNAs in HL-60 and U-937 cells.

Determination of WEE1 Expression Levels in Leukemia Cell Lines

Western blot analysis was conducted to examine endogenous WEE1 protein. To enrich for WEE1, which localizes to the nucleus, nuclear and cytoplasmic proteins were extracted from the same panel of cell lines used for quantification of *miR-17* and *miR-19a*. Western blot analysis revealed that, as expected, WEE1 is present in the nucleus (16) (Figure 4A), but not in the cytoplasm (Figure 4C). WEE1 expression from nuclear extracts was quantified

relative to β -Actin expression (Figure 4B). The cell lines with the highest relative expression of WEE1 were MonoMac6 and THP-1. Strikingly, the cell lines with the lowest relative expression of WEE1 were the same cell lines with the highest relative expression of *miR-17* and *miR-19a*, namely HL-60 and U-937. We conclude that in the cell lines tested, endogenous WEE1 protein expression is variable.

Determination of the Relationship between *miR-17-92* Expression and WEE1 Expression

We hypothesized that high endogenous expression of *miR-17-92* in leukemia cell lines would correspond with low endogenous expression of WEE1 in the same cell lines. Relative expression of *miR-17* that was determined by TaqMan MicroRNA Assays was plotted against relative expression of WEE1 that was determined by quantification of western blot analysis. A negative correlation was observed between the two variables with a coefficient of determination (R^2) of 0.6093 (Figure 5A). In general, as *miR-17* levels increased, there was a corresponding decrease in WEE1 expression levels. Similarly, a negative correlation was found between relative expression of *miR-19a* and relative expression of WEE1 with a coefficient of determination (R^2) of 0.5388 (Figure 5B). This result strengthens the conclusion that WEE1 is a valid target of the *miR-17-92* cluster.

Discussion

Identifying targets of the *miR-17-92* cluster is important for increased understanding of the complex gene expression pathways that are dysregulated in leukemia. Previous studies have experimentally validated a range of gene targets of the *miR-17-92* cluster in various systems. The individual miRNAs of this cluster have been shown to promote cell proliferation by downregulating p21 (17) and Pten (18), suppress apoptosis by downregulating E2Fs (19) and Bim (20), and induce angiogenesis in solid tumors by downregulating Tsp1 and CTGF (7). Since combined haploinsufficiency of Bim and Pten only partially mimics the oncogenic effects of *miR-17-92* overexpression (18), additional targets likely contribute to these effects. As such, we were interested in seeking novel targets of the *miR-17-92* cluster.

Bioinformatic miRNA target gene prediction algorithms were used to predict additional targets of *miR-17-92*. WEE1, a protein kinase that adds an inhibitory phosphate to Cdk1, was identified as a possible target of *miR-17*, *miR-20a*, *miR-18a*, *miR-19a*, and *miR-19b*. It was conceivable, therefore, that multiple miRNAs from the *miR-17-92* cluster collectively regulate WEE1 expression. We hypothesized that the cluster exerts combinatorial control on this target, thereby amplifying the effects of downregulation.

Luciferase reporter assays are considered the “gold standard” in validating miRNA gene targets (18, 21, 22). From our experiments, it appeared as though *miR-17* was the most crucial regulator of WEE1 expression from this cluster. The change in luciferase activity for *miR-17*, *miR-20a*, and *miR-18a* together was quite similar to the change in luciferase activity for *miR-17* alone, indicating that perhaps *miR-20a* and *miR-18a* are less effective at modulating WEE1. Furthermore, while the increase in luciferase activity for *miR-19a* and *miR-19b* was statistically significant, it was not as sizeable as for *miR-17*. Overall, from the luciferase reporter assays, we were able to experimentally confirm our hypothesis that WEE1 is a *bona fide* target of the *miR-17-92* cluster. Importantly, through mutagenesis

studies, we found the specific region in the 3' UTR of *WEE1* that *miR-17*, *miR-20a*, and *miR-18a* target (nucleotides 465 to 487) and the specific region in the 3' UTR of *WEE1* that *miR-19a* and *miR-19b* target (nucleotides 1069 to 1091).

TaqMan MicroRNA Assays are a useful technique for quantifying endogenous levels of miRNAs in tissues ranging from cell lines to patient samples (22). Previous studies have shown that the *miR-17-92* cluster is upregulated in samples from patients with acute leukemias bearing *MLL* rearrangements compared to patient samples with *inv(16)* or *t(8;21)* aberrations (9, 10). CHIP analysis showed that *MLL* fusion proteins upregulate expression of the *miR-17-92* cluster by directly binding to the locus promoter region (10). From these prior studies, we hypothesized that overexpression of *miR-17-92* would be observed in the *MLL* fusion leukemia cell lines tested here. All of the *MLL* fusion leukemia cell lines tested had increased *miR-17* and *miR-19a* expression as compared to the K562 leukemia cell line. However, HL-60 and U-937 displayed the highest expression of *miR-17* and *miR-19a* of the leukemia cell lines examined in the current study. This was not entirely unexpected because HL-60 cells have amplification of the *MYC* gene, which has been shown to directly transactivate transcription of the *miR-17-92* cluster (19). Likewise, U-937 cells are derived from lymphoma, and many lymphomas possess amplification of the human locus encoding the *miR-17-92* cluster (4). HL-60 and U-937 cell lines were not examined the earlier Chen study (10).

To further validate *WEE1* as a target of the *miR-17-92* cluster, western blot analysis was used to assess the relative endogenous expression of *WEE1* across a panel of cell lines. Remarkably, the two cell lines with the highest endogenous expression of *miR-17* and *miR-19a*, HL-60 and U-937, displayed the lowest endogenous expression of *WEE1*. *WEE1* expression was determined using western blot analysis because miRNA gene targeting always downregulates protein expression, but only in some instances downregulates mRNA expression. Thus, regardless of the mechanism for downregulation, mRNA cleavage or translational repression, protein expression would be affected. Messenger RNA cleavage takes place when the mRNA has extensive complementarity to the miRNA (2, 23). Translational repression occurs when there is insufficient complementarity for mRNA cleavage, but adequate complementarity at multiple sites within the 3' UTR for translational repression (2, 23). Translational repression is also the more common mechanism used by metazoan miRNAs. Since the individual miRNAs of the *miR-17-92* cluster are metazoan miRNAs and because there is not perfect complementarity between the miRNAs of the *miR-17-92* cluster and the 3' UTR of *WEE1*, *WEE1* protein levels were more likely to be affected.

We were interested to see if a relationship existed between endogenous *miR-17* or *miR-19a* expression and endogenous *WEE1* expression. Plotting the relative expression of *miR-17* versus the relative expression of *WEE1* revealed a negative correlation between the two variables. A similar negative correlation was observed for the relationship between the relative expression of *miR-19a* and the relative expression of *WEE1*. That is, as the expression of either one of the two miRNAs increased, the expression of *WEE1* decreased. We concluded that the regulation was likely occurring at physiological levels because we observed this trend while studying steady-state endogenous expression rather than by

overexpression. While the data indicates that there is a correlation between *miR-17-92* levels and WEE1 expression levels, it does not necessarily imply causation between the variables. However, causation was established through the luciferase reporter assays. Nevertheless, observing a negative correlation between *miR-17-92* and WEE1 in the cell lines we tested provides further evidence in support of WEE1 being a valid target of the *miR-17-92* cluster.

According to our model, when expression of the *miR-17-92* cluster is low, WEE1 is translated normally. As such, WEE1 functions to prevent cells from entering mitosis until they are ready. Conversely, when expression of the *miR-17-92* cluster is high, *miR-17*, *miR-20a*, or *miR-18a* bind to nucleotides 465 to 487 and *miR-19a* or *miR-19b* bind to nucleotides 1069 to 1091 of the 3' UTR of *WEE1*. It is conceivable that different molecules of *WEE1* mRNA have different combinations of miRNAs from the *miR-17-92* cluster bound at these target sites. Together with the RISC complex, the *miR-17-92* cluster represses translation of WEE1. WEE1 is a critical regulator of the G2/M transition, one of the restriction checkpoints. At each checkpoint, cell cycle progression stalls if flaws are detected. Because WEE1 inhibits cell cycle progression, it is an anti-proliferative protein. Consequently, downregulation of WEE1 by the *miR-17-92* cluster would have pro-proliferative functional effects. In fact, overexpression of *miR-17-92* has been shown to cause expansion of multipotent hematopoietic progenitors *in vivo* (24).

Recently, WEE1 has been identified as a possible therapeutic target in acute myeloid leukemia (25). The WEE1 inhibitor MK-1775 inhibited growth of various AML cell lines *in vitro* (26), and could synergize with cytarabine by preventing cytarabine-induced S-phase arrest (25). CHK1 activation could help overcome DNA damage caused by WEE1 inhibition, and combined inhibition of both WEE1 and CHK1 increased susceptibility of AML cells (26, 27). Furthermore, WEE1 inhibitor MK-1775 synergized with mTOR inhibition in KRAS-positive AML leukemias as well as in other mutant KRAS models, including lung cancer (28). A different WEE1 inhibitor, AZD1775, synergized with Vorinostat-mediated HDAC inhibition in AML with varied genetic mutations (29). It will be critical to determine which leukemia subtypes might best respond to WEE1 inhibitor therapies, and whether *miR-17-92* expression levels contribute to efficacy.

In summary, we have established WEE1 as a novel target of multiple miRs in the *miR-17-92* cluster. Moreover, we have determined the exact location within the 3' UTR of *WEE1* that *miR-17*, *miR-20a*, *miR-18a*, *miR-19a*, and *miR-19b* target. We further demonstrated a specific inverse relationship between endogenous *miR-17-92* expression and endogenous WEE1 expression in these cell lines.

It would be interesting to investigate the expression of WEE1 in samples from patients with different subtypes of leukemia to determine whether a negative correlation exists between *miR-17-92* and WEE1 in primary patient samples. This would also be of interest in other types of cancers including solid tumors. A positive finding would suggest that there are broader implications for this regulatory relationship which may be relevant for future therapeutic targeting.

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Figure 2. Luciferase Reporter and Mutagenesis Assays Validate *WEE1* as a *miR-17-92* Cluster Target

(A) Schematic of wild-type and mutant luciferase reporter constructs. (B)–(E) Relative luciferase activity measured 42 hours after the co-transfection of one of the luciferase reporter constructs with empty vector (B), or vectors expressing *miR-17* (C), *miR-17-19b* (D), or *miR-17-92* (E) along with *Renilla* luciferase-expressing construct in HEK293T cells. The data from three independent experiments is presented as relative firefly luciferase: *Renilla* luciferase activity normalized to the wild-type construct, with error bars showing standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

A **B**

Figure 3. Quantification of Endogenous *miR-17* and *miR-19a* Levels

Endogenous expression levels of *miR-17* (A) and *miR-19a* (B) in four MLL fusion leukemia cell lines (MV-4-11, RS4;11, THP-1, MonoMac6), three non-MLL fusion leukemia cell lines (K-562, HL-60, U-937), and one non-leukemia cell line (HEK293T). RNA was isolated from each cell line, cDNA was synthesized, and expression levels of *miR-17* and *miR-19a* were quantified with TaqMan MicroRNA Assays. Shown are average expression levels relative to *U6* snRNA and normalized to MV-4-11. Error bars indicate standard deviation.

Figure 4. Endogenous Nuclear and Cytoplasmic WEE1 Protein Expression

(A) Western blot analysis of endogenous WEE1 from nuclear extracts of the indicated cell lines. A shorter exposure (upper) and longer exposure (lower) of the WEE1 blot are shown. β-Actin served as a loading control. (B) Quantification of WEE1 expression levels from nuclear extracts relative to β-Actin levels. Relative expression was normalized to MV-4-11. Band intensity was quantified using MultiGauge V3.0 software. (C) Western blot analysis of endogenous WEE1 from cytoplasmic extracts of the same cell lines used in (A) and (B). β-Actin was used as a loading control.

A **B**

Figure 5. Negative Correlation between WEE1 Expression and *miR-17* or *miR-19a* Expression
Scatter plot of relative expression of WEE1 versus relative expression of *miR-17* (A) or *miR-19a* (B). Each point on the graph represents a unique cell line (MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T). The best fit line is shown as well as the equation of the line and the coefficient of determination (R^2).