WEE1 Is a Biological Target of the miR-17-92 Cluster in Leukemia

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LOYOLA UNIVERSITY CHICAGO

WEE1 IS A BIOLOGICAL TARGET OF
THE MIR-17-92 CLUSTER IN LEUKEMIA

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MOLECULAR BIOLOGY

BY
SONIA OLIKARA

CHICAGO, ILLINOIS
DECEMBER 2011
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To my family
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ABSTRACT

MicroRNAs (miRNAs, miRs) are 18-24 nucleotide single-stranded RNAs that bind to complementary sites in the 3’ untranslated region (UTR) of their mRNA targets. They act together with the RNA-induced silencing complex (RISC) to post-transcriptionally regulate target gene expression by either repressing translation or directly cleaving mRNA targets. The polycistronic miR-17-92 cluster, which encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a, is overexpressed in several solid tumors and hematopoietic malignancies. Recently, it was shown that miR-17-92 is overexpressed in leukemias arising from chromosomal translocations involving the Mixed Lineage Leukemia (MLL) gene. Since MLL fusion leukemias are categorized as being particularly aggressive, discovering targets of the miR-17-92 cluster may shed light on potential novel therapeutics.

In the present study, we used target gene prediction algorithms to predict potential targets of miRNAs within 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. We hypothesized that high miR-17-92 expression in MLL fusion leukemias causes downregulation of WEE1, which promotes cell cycle progression and contributes to leukemogenesis.

Through luciferase reporter assays and mutagenesis studies, we found 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. Surprisingly, we saw no significant increase in endogenous miR-17 or miR-19a expression as detected by TaqMan MicroRNA Assays in the established MLL fusion leukemia cell lines we tested (MV-4-11, RS4;11, THP-1, MonoMac6) as compared to the non-MLL fusion leukemia cell lines (K-562, HL-60, U-937). We believe that miR-17-92 expression levels may be dependent on factors in addition to the presence of a MLL fusion protein, especially in cell lines. Notably, while we did not detect a direct relationship between $MLL$ rearrangement status and miR-17-92 expression, we observed a negative correlation between endogenous miR-17 or miR-19a expression and endogenous $WEE1$ protein expression as detected by Western blot analysis in the same panel of cell lines. The results of this project suggest that $WEE1$ is a valid biological target of the miR-17-92 cluster in leukemia.
CHAPTER 1

LITERATURE REVIEW

Mixed Lineage Leukemia

The Mixed Lineage Leukemia (MLL) gene was first identified for its involvement in chromosomal translocations at chromosome 11, band q23 that cause acute lymphoid leukemias, acute myeloid leukemias, or mixed lineage leukemias, also known as myeloid-lymphoid leukemias\(^1\). Chromosomal rearrangements between nonhomologous chromosomes create an in-frame fusion between MLL and one of more than 60 different fusion partners\(^2\) (Figure 1A). A chimeric protein is produced in which the amino-terminus of MLL is fused with the carboxyl-terminus of the fusion partner\(^3\).

MLL fusion partner genes encode either nuclear proteins or cytoplasmic proteins, with different functions. Over 80% of MLL fusion leukemias possess a MLL fusion partner gene that encodes a nuclear protein such as AF4, AF9, ENL, ELL, AF10, and AF5q31\(^4\). Many of these nuclear fusion partners are normally present in a transcriptional elongation complex, while others are transcription factors. Many of the cytoplasmic fusion partners have the ability to dimerize or multimerize. Certain fusion partners are more prevalent in certain types of leukemia. For example, MLL-AF4 is more common in acute lymphoid leukemia\(^5\), while MLL-AF9 is more common in acute myeloid leukemia.

Leukemias arising from chromosomal translocations of the MLL gene account for 5 to 10% of all human acute leukemias\(^6\). MLL rearrangements are the most common
Figure 1. Mixed Lineage Leukemia

(A) Wild-type MLL and the MLL fusion protein after undergoing chromosomal translocation. (B) Event-free survival for patients with wild-type MLL and various MLL rearrangements. Figure was adapted from Pieters et al. 2007.10.
chromosome abnormalities found in infant leukemias and in patients previously treated with DNA topoisomerase II inhibitors\textsuperscript{7,8}. Compared to other leukemias, MLL fusion leukemias are particularly aggressive and have poor prognosis\textsuperscript{9,10}. Event-free survival of leukemia patients bearing \textit{MLL} translocations including t(9;11), t(4;11), and t(11;19) is significantly worse than for leukemia patients with wild-type \textit{MLL}\textsuperscript{10} (Figure 1B).

Wild-type MLL is a member of the Trithorax group of proteins. MLL and other Trithorax proteins are involved in maintaining active gene expression. These proteins antagonize the effect of Polycomb proteins, which are responsible for negatively regulating gene expression. \textit{Mll} homozygous null mice have defects in embryonic hematopoiesis and are embryonic lethal, suggesting a crucial role for MLL in development\textsuperscript{11}.

MLL is a very large protein that contains numerous functional domains\textsuperscript{3} (Figure 1A). Some domains are retained in the fusion protein including the AT-hooks and the repression domain. The AT-hooks bind AT-rich DNA\textsuperscript{12}. The repression domain contains a CXXC domain, which binds non-methylated CpG DNA\textsuperscript{13}. Other domains such as the four plant homeodomains (PHD), transcriptional activation domain, and histone methyltransferase domain (SET) are not retained in the fusion protein that causes leukemia. The individual MLL plant homeodomains, each of which fold into a structure that coordinates two zinc ions, have differing functions. Some are required for homodimerization and another for binding to cyclophilin Cyp33\textsuperscript{14}. The activation domain interacts with the CREB-binding protein (CBP), which recruits transcriptional
activators and possesses intrinsic histone acetyltransferase activity. The SET domain is responsible for trimethylation of lysine 4 on histone H3.

**MicroRNAs**

MicroRNAs (miRNAs, miRs) are approximately 22 nucleotide noncoding RNAs that function as antisense regulators of messenger RNAs (mRNAs). Victor Ambros first discovered this class of small RNAs in *C. elegans* in 1993. The *lin-4* miRNA was shown to regulate the transition from the first larval stage to the second by downregulating *lin-14* mRNA. In 2000, *C. elegans let-7* miRNA was shown to regulate the transition from the late larval stage to adult cell fates. Today over 500 miRNA genes have been discovered in the human genome alone with notably high degrees of sequence conservation across species.

Many miRNAs display distinct temporal and spatial expression patterns, while others are constitutively transcribed. RNA Polymerase II transcribes miRNA genes, which are located either within intergenic regions or within the introns of pre-mRNAs (Figure 2A). The transcribed pri-miRNA forms a stem loop structure. Drosha cleaves the ends of the stem to produce 60-70 nucleotide pre-miRNAs. Ran-GTP and Exportin5 facilitate active transport of the miRNA precursor from the nucleus to the cytoplasm. Dicer then cleaves off the loop to reveal a miRNA:miRNA duplex. Helicase unwinds the duplex and the miRNA strand whose 5’ end is more tightly paired is degraded. The other miRNA strand, which represents the mature miRNA, is loaded on the RNA-induced silencing complex (RISC), where it scans for perfect or near
Figure 2. Maturation of MicroRNAs and Mechanisms for Downregulating Target Gene Expression

(A) Maturation of miRNAs. miRNA genes are transcribed by RNA polymerase II and then cleaved by Drosha. The resulting pre-miRNA is transported into the cytoplasm, cleaved by Dicer, unwound by Helicase, and loaded onto the RISC complex. Figure was adapted from Bartel 2004. (B) and (C) Mechanisms utilized by miRNAs together with the RISC complex to downregulate target gene expression. Figures were adapted from Bartel 2004. (B) Extensive complementarity in the coding region or 3’ UTR specifies mRNA cleavage. (C) Short complementary segments in the 3’ UTR specifies translational repression.
perfect complementary mRNA sequences\textsuperscript{30}. The seed region, which encompasses nucleotides 2-7 of the miRNA, determines targeting of the miRNA to target mRNA\textsuperscript{31}.

miRNAs utilize different mechanisms to downregulate gene expression of their targets. When there is a high degree of complementarity to the mRNA sequence, the target mRNA is cleaved within the miRNA-binding site, which results in decreased mRNA and protein levels\textsuperscript{24} (Figure 2B). However, when there is a lower degree of complementarity to the mRNA sequence, translation is repressed, which results in decreased protein levels alone\textsuperscript{24} (Figure 2C). Translation is thought to be repressed by the slowing or stalling of ribosomes or by the degradation of newly synthesized polypeptide.

miRNAs are often abnormally expressed in many cancers including leukemias. For example, it was shown that MLL fusion proteins induce overexpression of miR-196b, which contributes to leukemogenesis\textsuperscript{32}. Determining which miRNAs are upregulated and downregulated in different subtypes of leukemia serves as a valuable diagnostic marker and may be useful in developing novel therapeutics\textsuperscript{33}. Techniques for replacing underexpressed miRNAs or inhibiting overexpressed miRNAs both \textit{in vitro} and \textit{in vivo} are currently being developed.

\textbf{miR-17-92 Cluster}

Although many miRNAs are present alone in the genome, some are located in clusters containing multiple miRNAs. miRNA clusters are expressed together as a long precursor and are then processed into individual miRNAs\textsuperscript{34}. The miR-17-92 cluster is located within an 800 base-pair region of chromosome 13 within the third intron of a
primary transcript called C13orf25\textsuperscript{35} (Figure 3A). The only known function of this transcript is to produce these miRNAs. The miR-17-92 cluster encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a)\textsuperscript{36}. The miRNAs of the miR-17-92 cluster share a great deal of sequence homology, suggesting that they might be able to act collectively on a common target. Gene duplications have produced miR-106a-363 and miR-106b-25, two paralogs of the miR-17-92 cluster\textsuperscript{36}. miRNAs from these three clusters are placed into families based on conserved seed sequences\textsuperscript{31} (Figure 3B).

The miR-17-92 cluster is crucial for normal development of the heart, lungs, and immune system. Knockout of this cluster has been shown to result in smaller embryos and immediate postnatal death due to ventricular septal defects in the heart and severely hypoplastic lungs\textsuperscript{37}. It has been demonstrated that the miR-17-92 cluster is highly expressed in embryonic lung tissue, but decreases over time\textsuperscript{38}. Additionally, this cluster is involved in promoting progression from pro-B to pre-B cells\textsuperscript{37}.

The miR-17-92 cluster acts as an oncogene\textsuperscript{39} and is overexpressed in hematopoietic malignancies and solid tumors including those originating from the breast, colon, lung, pancreas, prostate, and stomach\textsuperscript{40}. Several target genes with important roles in cell cycle progression, apoptosis, and angiogenesis have been identified and experimentally validated for this cluster. The E2F family of transcription factors, which when expressed at high levels can induce apoptosis, is one such target\textsuperscript{41}. The miR-17-92 cluster suppresses apoptosis by downregulating E2F1, E2F2, and E2F3. The pro-apoptotic gene \textit{Bim} has also been shown to be a direct target of the miR-17-92 cluster\textsuperscript{42}. 
Figure 3. The miR-17-92 Cluster

(A) The miR-17-92 cluster is located within an intron on chromosome 13. Figure was adapted from Mendell 200836. (B) miRNAs are aligned to show nucleotide sequence conservation. Nucleotides that are conserved within a miRNA family are highlighted in blue. The seed sequences (nucleotides 2-7) of each miRNA are in bold print.
Another target that has been validated is the cyclin-dependent kinase inhibitor p21, which is a negative regulator of the G1/S checkpoint\textsuperscript{43}. The miR-17-92 cluster is also known to downregulate expression of the tumor suppressor Pten\textsuperscript{44}. Finally, the anti-angiogenic proteins Tsp1 and CTGF are both negatively regulated by the miR-17-92 cluster\textsuperscript{45}. High levels of the miR-17-92 cluster have been shown to increase the number of leukemia stem cells, block differentiation, and enhance proliferation, while low levels of the miR-17-92 cluster increase differentiation and decrease self-renewal\textsuperscript{46}.

Each subtype of acute myeloid leukemia and acute lymphoid leukemia has a unique miRNA expression profile. 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\textsuperscript{47, 48}. Furthermore, this cluster is overexpressed in mouse leukemia cells with MLL-ELL and MLL-ENL fusions, although the expression is not quite as high as in the corresponding human leukemias\textsuperscript{49}.

**WEE1**

Wee1 was first discovered in the fission yeast *S. pombe* as a mitotic inhibitor that controls the G2/M transition\textsuperscript{50}. Fission yeast deficient in Wee1 prematurely enter mitosis and divide at a small size. The kinase was named “Wee” in reference to the small size of fission yeast lacking Wee1. The catalytic domain of the human gene was cloned in 1991\textsuperscript{51}, and the full-length gene was identified in 1995\textsuperscript{52}. Wee1 has also been found in *S. cerevisiae, Xenopus, Drosophila*, and *C. elegans*. 
Human WEE1 is a protein kinase that adds an inhibitory phosphate on Tyr15 of Cyclin dependent kinase 1 (Cdk1) during interphase. 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.

As shown in Figure 4, the pathway from inactive Cdk1 to fully active Cdk1 requires several molecular players. During interphase, Cdk1 associates with Cyclin B and is phosphorylated by Cdk-activating kinase (CAK) on Thr161. Simultaneously, WEE1 phosphorylates Cdk1 on Tyr15, while the WEE kinase family member, MYT1, phosphorylates Cdk1 on Thr14 and Tyr15. These inhibitory phosphorylations on Thr14 and Tyr15 dominantly inhibit Cdk1 until the onset of mitosis, at which point Cdc25 dephosphorylates Thr14 and Tyr15. Cdk1 becomes active and triggers the rapid switch into mitosis. Upon entry into mitosis, WEE1 is inactivated by phosphorylation on Ser53 and Ser123 by Plk1 and Cdk1. WEE1 is subsequently ubiquitinated and degraded. WEE1 levels remain low throughout M phase and G1 phase.

The WEE family of kinases consists of WEE1, WEE2, and MYT1. While WEE1 and WEE2 solely phosphorylate Cdk1 on Tyr15, MYT1 is a dual-specificity kinase that phosphorylates Cdk1 on both Thr14 and Tyr15. WEE1 and WEE2 are soluble proteins that localize to the nucleus. Conversely, MYT1 is membrane-associated and localizes to the cytoplasmic endoplasmic reticulum and Golgi apparatus. Importantly, in *Xenopus*, it was found that Wee1 is 10-fold more active than Wee2.
During interphase, Cdk1 associates with Cyclin B. Cdk-activating kinase (CAK) then adds an activating phosphate to Cdk1 on Thr161. WEE1 adds an inhibitory phosphate to Cdk1 on Tyr15, while MYT1 adds two inhibitory phosphates to Cdk1 on Thr14 and Tyr15. At the onset of mitosis, Cdc25 phosphatase removes the inhibitory phosphates, thereby fully activating Cdk1 and promoting mitosis.
The kinase domain of WEE1 is highly conserved across WEE family members. All WEE family members possess five conserved amino acid residues within their kinase domain that are unique to this family of kinases\textsuperscript{58}. The noncatalytic regions of these proteins are important for regulation, protein-protein interactions, and subcellular localization.

miR-195 was recently shown to target two sites in the 3’ UTR of *WEE1* in human embryonic stem cells\textsuperscript{59}. Since WEE1 levels control the rate of human embryonic stem cell division, downregulation of WEE1 by miR-195 promotes the cell cycle and increases cell proliferation\textsuperscript{59}. This study was the first to show a miRNA regulating WEE1. Nevertheless, it is still important to discover novel miRNAs that target WEE1. It is conceivable that in different types of cells, other unique miRNAs target WEE1 for downregulation. For this reason, we set out to validate WEE1 as a target of the miR-17-92 cluster.
CHAPTER 2

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 Wild-Type Luciferase Reporter Construct

In order to amplify the 3’ UTR of WEE1 (NM_003390.3), the following PCR was set up: 40.1 uL dH2O, 5 uL 10x Cloned Pfu buffer, 0.4 uL 25 mM dNTPs, 1 uL 1ug/uL human genomic DNA, 1.25 uL 10 uM Forward Primer (Table 1), 1.25 uL 10 uM Reverse Primer (Table 1), 1 uL Cloned Pfu DNA Polymerase (Stratagene, Catalog #600159-81). The PCRs were run in the PCR Express Thermal Cycler (Hybaid) according to the following protocol: one cycle of 95°C for 45 seconds, 40 cycles of 95°C for 45 seconds, 62.6°C for 45 seconds, 72°C for 3 minutes, one cycle of 72°C for 10 minutes, and hold at 4°C. The band at 939 bp was cut out and gel extracted using the QIAEX II Gel Extraction Kit (Qiagen, Catalog #20051).

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<th>Amplicon</th>
<th>Primers (5’ to 3’)</th>
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<tr>
<td><strong>WEE1 3’ UTR</strong>&lt;br&gt;(nucleotides 2418 - 3356 of NM_003390.3)</td>
<td>Forward: ATGTTACACCAGCCTTTCCAGGGT&lt;br&gt;Reverse: AGACAATTAAGGTAAGCTCAGAGTGA</td>
</tr>
</tbody>
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In order to add restriction sites to the ends of the above PCR product to facilitate cloning into the reporter vector, the following PCR was set up: 40.1 uL dH2O, 5 uL 10x Cloned Pfu buffer, 0.4 uL 25 mM dNTPs, 1 uL of the above PCR product, 1.25 uL 10 uM Forward Primer (Table 2), 1.25 uL 10 uM Reverse Primer (Table 2), 1 uL Cloned Pfu DNA Polymerase. The PCRs were run according to the following protocol: one cycle of 95°C for 45 seconds, 40 cycles of 95°C for 45 seconds, 65.2°C for 45 seconds, 72°C for 3 minutes, one cycle of 72°C for 10 minutes, and hold at 4°C. The band at 969 bp was cut out and gel extracted.

**Table 2. Primers used to add flanking restriction sites to the 3’ UTR of WEE1**

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<th>Amplicon</th>
<th>Primers (5’ to 3’)</th>
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<tr>
<td>WEE1 3’ UTR</td>
<td>Forward: TCTCTCTCTACTAGTATGTTACACCAGCCTTTCCAGGGT</td>
</tr>
<tr>
<td>(nucleotides 2418SpeI - 3356HindIII)</td>
<td>Reverse: TCTCTCTCTAAGCTTAGACAATTAAGGTAAGCTCAGAGTGA</td>
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The 3’ UTR of WEE1 that was now flanked by SpeI and HindIII restriction sites was digested with SpeI and HindIII at 37°C for 2 hours along with the pMIR-REPORT Luciferase vector (Applied Biosystems, Catalog #AM5795). The gel extracted digests were then ligated using T4 DNA Ligase (New England Biolabs, Catalog #M0202L) at 16°C overnight to create a construct with the 3’ UTR of WEE1 immediately following the luciferase gene. The ligation was electroporated into homemade DH10β Electrocompetent Cells using 1 uL ligation reaction and 20 uL cells using the following electroporator settings: 4 kΩ resistance, 330 µF capacitance. The transformants were miniprepped using the GeneJET Plasmid Miniprep Kit (Fermentas, Catalog #K0503). The clone was confirmed by sequencing (ACGT, Inc.).
Cloning of Mutant Luciferase Reporter Constructs

In order to mutate the putative miR-17-92 binding sites in the 3’ UTR of *WEE1*, three mutant luciferase reporter constructs were generated. For one of the constructs, the putative miR-17, miR-20a, and miR-18a target site was mutated. In another of the constructs, the putative miR-19a and miR-19b target site was mutated. The third mutant construct had all the putative miR-17-92 target sites mutated.

To produce the construct with the putative miR-17, miR-20a, and miR-18a target site mutated (referred to as miR-17 Mut), a modified site-directed mutagenesis protocol with non-overlapping primers was used to set up the following PCR using an error proof polymerase: 32.5 uL dH2O, 10 uL 5x Phusion HF Buffer, 1 uL 10 mM dNTPs, 2.5 uL 10 uM Forward Primer (Table 3), 2.5 uL 10 uM Reverse Primer (Table 3), 1 uL 10 ng/uL template (wild-type luciferase reporter construct), 0.5 uL Phusion DNA Polymerase (Finnzymes, Catalog #F-530S). The PCRs were run according to the following Touch Down PCR protocol: one cycle of 98°C for 30 seconds, 20 cycles of 98°C for 10 seconds, touch down from 60°C to 50°C in 0.5°C increments for 30 seconds, 72°C for 8 minutes, 25 cycles of 98°C for 10 seconds, 50°C for 30 seconds, 72°C for 8 minutes, one cycle of 72°C for 10 minutes, and hold at 4°C. The PCR product was DpnI treated at 37°C for 1 hour to digest the parental DNA template. It was then electroporated into DH10β Electrocompetent Cells using 1 uL PCR product and 20 uL cells. The transformants were miniprepped, and the plasmid DNA was sequenced. Since stray mutations can be introduced during site-directed mutagenesis, the mutated region was ligated into the
pMIR-REPORT Luciferase vector using the SpeI and HindIII restriction sites used previously.

Table 3. Primers used to mutate the putative miR-17, miR-20a, and miR-18a target site in the 3’ UTR of WEE1

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primers (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase WEE1 3’ UTR miR-17 Mut pMIR-REPORT</td>
<td>Forward: GACTTGTATATCCCACTGGGAGACAGGGGTAGGCATTGCA TGAACCATGGGATG Reverse: GCCAATCAATGTTAATAAAAACACAAGTCAAGACAGAATGTA CCACATGTTTAGACC</td>
</tr>
</tbody>
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To generate the construct with the putative miR-19a and miR-19b target site mutated (referred to as miR-19 Mut) and the construct with all the putative miR-17-92 target sites mutated (referred to as miR-17,19 Mut), the following PCRs were set up: 39.5 uL dH2O, 5 uL 10x Cloned Pfu buffer, 1 uL 10 mM dNTPs, 1 uL 10 ng/uL template (wild-type luciferase reporter construct or miR-17 Mut), 1.25 uL 10 uM Forward Primer (Table 4), 1.25 uL 10 uM Reverse Primer (Table 4), 1 uL Cloned Pfu DNA Polymerase. The PCRs were run according to the following protocol: one cycle of 95°C for 45 seconds, 40 cycles of 95°C for 45 seconds, gradient from 55°C to 70°C for 45 seconds, 72°C for 2 minutes, one cycle of 72°C for 10 minutes, and hold at 4°C. The PCR products from all three annealing temperatures were pooled together and PCR purified. Each of the inserts and the pMIR-REPORT Luciferase vector were digested with SpeI and HindIII at 37°C for 2 hours. The gel extracted digests were then ligated overnight at 16°C using a 3:1 molar ratio of insert to vector. The ligations were electroporated into homemade DH10β Electrocompetent Cells using 2 uL ligation reaction and 20 uL cells. The transformants were miniprepped and all clones were confirmed by sequencing.
Table 4. Primers used to mutate the putative miR-19a and miR-19b target site in the 3’ UTR of WEE1

<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primers (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>Luciferase WEE1 3’ UTR miR-19 Mut pMIR-REPORT</td>
<td>Forward: TCTCTCTCTACTAGTATGTTACACCAGGTTTTTCCAGGGT&lt;br&gt;Reverse: CTTTTATTTAAGCTTAGACATATTTAAGGTAAGCTCAGAGTGAC TTATAATGCCAATCAATGTTAATTAAAACACAAGTCAAGCAAT GTACACCATGTTTTAGACC</td>
</tr>
<tr>
<td>Luciferase WEE1 3’ UTR miR-17,19 Mut pMIR-REPORT</td>
<td>Forward: TCTCTCTCTACTAGTATGTTACACCAGGTTTTTCCAGGGT&lt;br&gt;Reverse: CTTTTATTTAAGCTTAGACATATTTAAGGTAAGCTCAGAGTGAC TTATAATGCCAATCAATGTTAATTAAAACACAAGTCAAGCAAT GTACACCATGTTTTAGACC</td>
</tr>
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Luciferase Reporter Assays

The wild-type and mutant luciferase reporter constructs as well as miR-17 MSCV-PIG (Jianjun Chen, University of Chicago), miR-17-19b MSCV-PIG (J. Chen), miR-17-92 MSCV-PIG (J. Chen), MSCV-PIG (J. Chen), and pRL-TK (Promega, Catalog #e2241) were midiprepped using the Plasmid Midi Kit (Qiagen, Catalog #12145).

60,000 HEK293T cells were plated into each of 16 wells of a 24-well plate (TPP, Catalog #92024). Each well was transfected 24 hours later using 6 ng control vector (pRL-TK), 120 ng Luciferase WEE1 3’ UTR (Wild-Type, miR-17 Mut, miR-19 Mut, or miR-17,19 Mut), and 600 ng miRNA (miR-17, miR-17-19b, miR-17-92, or Empty Vector). The CalPhos Mammalian Transfection Kit (Clontech, Catalog #631312) was used for the transfections. The medium was removed from each well the following day and replaced with fresh complete medium (DMEM (HyClone), 10% FBS, and 1% Pen/Strep).

The reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Catalog #E1910). 42 hours after transfection, the medium was removed from each well of the 24-well plate and the cells were gently washed with 200 uL sterile 1x PBS. 100 uL 1x Passive Lysis Buffer (1600 uL dH2O, 400 uL 5x Passive
Lysis Buffer) was added to each well and the plate was rocked on a rocking platform at room temperature for 15 minutes. Since the experiment was performed in triplicate, three 20 uL aliquots of each of the 16 cell lysates were transferred to a white round-bottom 96-well plate (Costar). The plate was centrifuged at 1500 rpm for 5 minutes at room temperature to bring the cell lysates to the bottom of the wells.

Using the Microplate Luminometer (Veritas), both injectors were flushed three times with dH$_2$O, three times with 70% Ethanol, three times with dH$_2$O, and then three times with air. The first injector was primed with Luciferase Assay Reagent II (lyophilized Luciferase Assay Substrate in Luciferase Assay Buffer II) and the second injector was primed with Stop & Glo Reagent (48 uL 50x Stop & Glo Substrate, 2352 uL Stop & Glo Buffer). The assay was run using the luminometer, which dispensed 100 uL Luciferase Assay Reagent II into each well, measured firefly luciferase, dispensed 100 uL Stop & Glo Reagent into each well, and measured *Renilla* luciferase. After the run, both injectors were flushed again. 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**

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) were thawed from liquid nitrogen. 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. Each cell line was expanded from a 10 cm² flask to two 75 cm² flasks. The cell pellets were resuspended in 3 mL sterile 1x PBS and then counted using the Bright Line Counting Chamber (Hausser Scientific Company).

For TaqMan MicroRNA Assays, 2,500,000 cells were aliquoted per microfuge tube for each cell line. The cell pellets were resuspended in 500 uL TRI Reagent (Sigma, Catalog #T9424) by repeated pipetting. The resuspended cell lysates were stored at -80°C until RNA was isolated.

For Western blotting, 1,000,000 cells were aliquoted per microfuge tube for each cell line. Cell pellets were incubated on ice for immediate use in nuclear and cytoplasmic protein extraction.

RNA Isolation and cDNA Synthesis

RNA was isolated from MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T cells according to the manufacturer’s protocol (Sigma). To summarize, cells that had been resuspended in TRI Reagent and stored at -80°C were briefly thawed in a room temperature water bath. The samples were then allowed to stand at room temperature for 5 minutes. 100 uL of chloroform was added to each sample and the tubes were shaken vigorously for 15 seconds. The samples were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 15 minutes at 4°C. The colorless aqueous phase (upper layer) containing the RNA was transferred to a fresh microfuge tube by pipetting. 250 uL of isopropanol was added
and mixed. The samples were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 minutes at 4°C. 500 uL of 75% ethanol was added to the RNA pellet and vortexed. The tubes were centrifuged at 7,500 x g for 5 minutes at 4°C. The supernatant was discarded and then the RNA pellet was air-dried in the hood for 10 minutes. The RNA pellet was reuspended in 20 uL of RNase-free water. The tube was incubated in a 55°C water bath for 10 minutes with periodic mixing. The isolated RNA was stored at -80°C.

cDNA was reverse transcribed from total isolated RNA using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Catalog #4366596) and primers specific to each miRNA from the TaqMan MicroRNA Assay Kit (Applied Biosystems). 10 ug of RNA was used for each reaction. For each 15 uL reverse transcription reaction, the following components were combined on ice: 0.15 uL 100 mM dNTPs (with dTTP), 1 uL MultiScribe Reverse Transcriptase (50 U/uL), 1.5 uL 10x Reverse Transcription Buffer, 0.19 uL RNase Inhibitor (20 U/uL), 4.16 uL Nuclease-free water, 5 uL total RNA diluted to a concentration of 2 ng/uL (isolated from either MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, or HEK293T cells), and 3 uL RT primer (specific to either U6 snRNA (constitutively expressed control), miR-17, or miR-19a). The reactions were incubated on ice for 5 minutes and subsequently loaded in the PCR Express Thermal Cycler (Hybaid). The following program was run: 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes, and hold at 4°C. The cDNA was stored at -20°C until the Real-Time PCR step.
TaqMan MicroRNA Assays

During the PCR amplification step, AmpliTaq Gold DNA polymerase was used to amplify target cDNA using sequence-specific primers. For each 20 uL reaction, the following components were combined on ice: 10 uL TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Catalog #4324018), 7.67 uL Nuclease-free water, 1 uL 20x TaqMan MicroRNA Assay mix (specific to either U6 snRNA, miR-17, or miR-19a), and 1.33 uL of the cDNA from the reverse transcription step. Since reactions were performed in triplicate, 20 uL of complete PCR master mix was dispensed into each of three wells of a clear 96-well PCR plate. The plate was sealed with an optical adhesive cover and centrifuged at 1500 rpm for 5 minutes. Real-time PCR was performed using the ABI 7300 Real-Time PCR System (Applied Biosystems) using the following program: one cycle of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. 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 $2^{-\Delta\Delta Ct}$ method. 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. 1,000,000 cell aliquots from MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T cells that were pelleted according to the protocol above were resuspended in 400 uL 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. 25 uL of 10% Nonidet NP-40 (Calbiochem, Catalog #492015) was added and then 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 in 30 uL aliquots and stored at -80°C until further analysis. The nuclear pellet was resuspended in 50 uL 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 vigorously rocked for 15 minutes on a rotator (Glas-Col) 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 in 30 uL aliquots and stored at -80°C until further analysis.

**Western Blot Analysis**

SDS-polyacrylamide (10%) gels (10% Bottom-Resolving; 4% Top-Stacking) with ten wells each were poured. The protein samples were prepared by combining 30 uL cytoplasmic or nuclear extracts with 10 uL Sample Loading Buffer (2.5 mL 1 M Tris pH 6.8, 1 g SDS, 0.05 g Bromophenol Blue, 5 mL Glycerol, 2.5 mL H2O, 500 mM DTT). The samples were vortexed, boiled at 95°C for 5 minutes, briefly centrifuged, and then loaded in the wells. 10 uL of Precision Plus Protein Kaleidoscope Standard (Bio-Rad, Catalog #161-0375) was loaded as a marker. The gels were electrophoresed at 150 V, 0.5 A for 65 minutes using 1x Running Buffer (5x Running Buffer: 15.1 g Tris base, 94 g Glycine, 50 mL 10% SDS, final volume brought up to 1 L using dH2O).
Immobilon Transfer Membranes (Millipore, Catalog #IPVH00010) were pre-incubated with methanol for 5 minutes and then washed twice with dH2O. The membranes and gels were then equilibrated in 1x CAPS Buffer (100 mL 10x CAPS Buffer (22.13 g CAPS, final volume brought up to 1 L using dH2O, pH 11.0), 100 mL Methanol, 800 mL dH2O) for 25 minutes. Gels were transferred to membranes at 70 V, 0.5 A for 2 hours using 1x CAPS Buffer.

The membranes were each blocked in 5% blocking solution (TBS-T (75 mL 2 M NaCl, 20 mL 1 M Tris pH 7.5, 904 mL dH2O, 1 mL Tween-20), 5% w/v BSA) for 1 hour at room temperature on the Belly Dancer (Stovall Life Science). The membranes were then incubated in primary antibody mixture (TBS-T, 5% w/v BSA, WEE1 rabbit polyclonal antibody (Cell Signaling, Catalog #4936, 1:1000 dilution)) with gentle shaking on the Orbitron Rotator II (Boekel Scientific) at 4°C overnight. The membranes were washed three times for 10 minutes each with 15 mL TBS-T. The membranes were then incubated in secondary antibody mixture (TBS-T, 5% w/v BSA, ECL Anti-Rabbit IgG-HRP Antibody (GE Healthcare, Catalog #NA934V, 1:3000 dilution)) for 2 hours at room temperature on the Belly Dancer. The membranes were washed five times for 15 minutes each with 15 mL TBS-T.

Using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Catalog #34078), the Stable Peroxide Solution and the Luminol Enhancer Solution were mixed at a 1:1 ratio to prepare the substrate working solution. The membranes were incubated with substrate working solution for 5 minutes at room temperature on the Belly Dancer. The excess reagent was drained and the membranes
were wrapped in clear plastic wrap. The membranes were developed using the FujiFilm
LAS-3000 Luminescent Image Analyzer with Image Reader LAS-3000 software.

In order to normalize for loading, the membranes were washed two times for 5
minutes each with 15 mL TBS-T and then stripped by performing two 10 minute
incubations with 15 mL 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 subsequently
washed two times for 10 minutes each with 15 mL PBS followed by two washes for 5
minutes each with 15 mL TBS-T. The membranes were blocked with 5% blocking
solution as described above and then incubated with the appropriate primary and
secondary antibody mixtures. β-Actin mouse monoclonal antibody (Sigma, Catalog
#A5441, 1:5000 dilution) was used as the loading control primary antibody followed by
ECL Anti-Mouse IgG-HRP Antibody (GE Healthcare, Catalog #NA931V, 1:3000
dilution). 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 MV-4-11 expression levels. The experiment was repeated twice.
CHAPTER 3

RESULTS

Aim: Determine whether WEE1 is a \textit{bona fide} target of the miRNAs within the miR-17-92 cluster in MLL fusion leukemias

It was recently shown that overexpression of the miR-17-92 cluster is a signature of MLL fusion leukemias\textsuperscript{48}. Because leukemias caused by rearrangement of the \textit{MLL} gene are particularly aggressive and have poor prognosis, we predict that identification of mRNA transcripts targeted for downregulation by this miRNA cluster will shed light on novel molecular targets for this disease.

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. TargetScan predicts targets by searching for sites within human untranslated regions that match the seed region of miRNAs. MicroCosm Targets functions by identifying high complementarity between miRNAs and 3’ UTRs while favoring complementarity in the seed region. PicTar predicts targets of human miRNAs based on conservation of the target sequence across species. miBridge searches for both 5’ UTR and 3’ UTR target sites in the same mRNA.

For each miRNA of the cluster, we compiled a list 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. The data was synthesized to
assemble a list of gene targets that appeared in more than one prediction program. 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 5A. 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 target site. The predicted alignment between WEE1 mRNA and each of the individual miRNAs of the cluster shows that there is a high degree of sequence complementarity within each paring especially in the seed region (Figure 5B). We hypothesized that the cluster exerts combinatorial control on this target, thereby amplifying the effects of downregulation. The goal of this aim is to elucidate whether WEE1 is a valid target of miR-17-92 in MLL fusion leukemias.

**Determination of whether miR-17-92 Specifically Targets the 3’ Untranslated Region of WEE1**

A study published earlier this year (after my project had been initiated) demonstrated that relative luciferase activity decreased by 21% when a luciferase reporter construct containing the 3’ UTR of WEE1 was co-transfected with miR-17 compared to when a luciferase reporter construct without the 3’ UTR was co-transfected with miR-17\(^1\). Similarly, the authors found a 15% reduction in luciferase activity with miR-20a\(^1\). While these results suggest that WEE1 is a target of miR-17 and miR-20a, they fail to identify the specific region of binding within the 3’ UTR and also neglect to investigate whether the rest of the miR-17-92 cluster exerts a regulatory effect on WEE1.
Figure 5. Predicted miR-17-92 Binding Sites in the 3’ UTR of WEE1

(A) Sequence of WEE1. The protein coding sequence is indicated in blue and the 3’ UTR is the region from the stop codon to the poly-A tail. The putative miR-17-92 target sites are highlighted in yellow. (B) Predicted alignment between WEE1 mRNA and miR-17, miR-20a, miR-18a, miR-19a, and miR-19b. The microRNA seed sequence is indicated in pink.
To establish whether WEE1 is a biological target of the entire miR-17-92 cluster and to determine the specific regions being targeted, we performed a luciferase reporter assay along with mutagenesis studies. 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 gene (Figure 6A). We also generated three mutant versions of the reporter construct that 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. Each guanine was converted to a thymine and vice versa. Each cytosine was converted to an adenine and vice versa. 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 6A). The third mutant has all the putative miR-17-92 target sites mutated (Figure 6A).

We co-expressed the wild-type or one of three mutant reporter constructs with miR-17, miR-17-19b, miR-17-92, or empty vector in HEK293T cells. Renilla luciferase, which was under the control of the HSV TK promoter, was also co-expressed for each condition as a control. 42 hours after transfection, the cells were lysed and firefly and Renilla luciferase activity were measured. Firefly luciferase activity was normalized relative to Renilla luciferase activity to control for differences in transfection efficiency. Results are presented from one representative experiment performed in triplicate. Three independent experiments were conducted.
Figure 6. Luciferase Reporter and Mutagenesis Assays

(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 reporter constructs with miR-17 (B), miR-17-19b (C), miR-17-92 (D), or empty vector (E) along with Renilla luciferase in HEK293T cells.  The data is presented as relative firefly luciferase: Renilla luciferase normalized to the wild-type construct, with error bars showing standard deviation.  * p<0.05; ** p<0.01; *** p<0.001.
Since the alignment score between WEE1 and the individual miRNAs of the miR-17-92 cluster is high, we hypothesized that WEE1 is a physiological target of miR-17-92. If WEE1 is in fact a target, when the wild-type reporter construct is co-expressed with any of the miRNAs of the cluster, the miRNA will bind to the 3’ UTR and repress translation of luciferase, resulting in low luminescence. On the other hand, co-expression of a reporter plasmid containing mutated binding sites with one of the miRNAs of the cluster will inhibit binding and result in relatively higher luminescence.

As seen in Figure 6B, 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). There is a statistically significant increase in luciferase activity (p<0.001) in both cases. This indicates that WEE1 is a valid target of miR-17. Because luciferase activity does not increase when the putative miR-19a and miR-19b site is mutated, we can deduce that miR-17 specifically targets the predicted miR-17 target site (Figure 6B).

The miR-17-19b construct expresses all of the miRNAs present in the miR-17-92 cluster, except for miR-92a. All five of the miRNAs within miR-17-19b are 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 6C). This suggests that WEE1 is a target of at least one of these miRNAs. Notably, the change in luciferase activity is quite similar to the change in
luciferase activity for miR-17 alone, indicating that perhaps miR-20a and miR-18a are less important than miR-17 in regulating WEE1. 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 6C). This implies that WEE1 is a target of miR-19a, miR-19b, or both. Finally, when all of the predicted target sites are mutated, co-expression of all five miRNAs increases luciferase activity by a larger margin, indicating that possibly all five miRNAs collectively regulate WEE1 expression (Figure 6C).

As shown in Figure 6D, 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. Since the reporter construct with all of the predicted target sites mutated had the greatest increase in luciferase activity relative to the wild-type construct, this provides further evidence that the entire miR-17-92 cluster is acting jointly to downregulate WEE1 expression.

Surprisingly, there were small, but statistically significant increases in luciferase activity in the presence of the empty vector (Figure 6E). This observation led us to predict that high endogenous miR-17-92 levels in HEK293T cells could be exerting an effect on this assay. It was later confirmed by TaqMan MicroRNA Assays that HEK293T cells have relatively high endogenous levels of miR-17 and miR-19a (Figure 7A, B).

From the luciferase reporter and mutagenesis assays, we can conclude that miR-17 specifically targets nucleotides 465 to 487 of the 3’ UTR of WEE1, which leads to
downregulation of WEE1. miR-20a and miR-18a also conceivably 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 MLL Fusion Leukemia Cell Lines Compared to Non-MLL Fusion Leukemia Cell Lines**

Previous studies have shown that the miR-17-92 cluster is particularly upregulated in samples from patients with acute leukemias bearing MLL rearrangements compared to patient samples with other common translocations\(^{47,48}\). ChIP analysis showed that MLL fusion proteins upregulate expression of the miR-17-92 cluster by directly binding to the locus promoter region\(^{48}\). From these prior studies, we hypothesized that overexpression of miR-17-92 would also be a signature of MLL fusion leukemia cell lines.

To quantify endogenous miR-17-92 expression in MLL fusion leukemia cell lines and non-MLL fusion leukemia cell lines, we isolated RNA, synthesized cDNA using a looped reverse transcription primer specific to each miRNA, and performed TaqMan MicroRNA Assays using a variety of cell lines. The MLL fusion leukemia cell lines included those with MLL-AF4 fusions (MV-4-11 and RS4;11) and MLL-AF9 fusions (THP-1 and MonoMac6). These cell lines represent the most common MLL translocations. The non-MLL fusion leukemia cell lines chosen included one with a BCR-ABL gene fusion (K-562), one with amplified c-Myc (HL-60), and a histiocytic lymphoma (U-937). We also included a non-leukemia cell line (HEK293T) as a control.
We selected two representative miRNAs, miR-17 and miR-19a, to quantify. miR-17 was chosen 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, since miR-19a and miR-19b differ by only one nucleotide and share the same target site, miR-19a was selected as the second representative miRNA. Ubiquitously expressed U6 snRNA served as the internal control. The assay was performed in triplicate and repeated two to five times.

Based on results from the bead-based miRNA expression profiling assay and the TaqMan qPCR assay\textsuperscript{47, 48}, we expected the MLL fusion leukemia cell lines to have high levels of miR-17 and miR-19a and the non-MLL fusion leukemia cell lines and the non-leukemia control cell line to have low expression of these two miRNAs.

Surprisingly, our results showed no significant increase in miR-17 (Figure 7A) or miR-19a (Figure 7B) expression in the MLL fusion leukemia cell lines compared to the non-MLL fusion leukemia cell lines and the non-leukemia control. The cell lines with the highest expression of these two miRNAs were HL-60 and U-937 cells, both of which are non-MLL fusion leukemia cells.

From the TaqMan MicroRNA Assays, we can conclude that miR-17-92 expression levels in leukemia cell lines are dependent on factors in addition to the presence of a MLL translocation.

**Determination of WEE1 Expression Levels in MLL Fusion Leukemia Cell Lines Compared to Non-MLL Fusion Leukemia Cell Lines**

Western blot analysis was conducted to examine endogenous WEE1 protein levels in MLL fusion leukemia cell lines and non-MLL fusion leukemia cell lines. To
(A) 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.
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 levels (MV-4-11, RS4;11, THP-1, MonoMac6, K-562, HL-60, U-937, and HEK293T). Nuclear and cytoplasmic extracts were electrophoresed on SDS-polyacrylamide gels, transferred to PVDF membranes, and probed for WEE1 expression using an antibody that is reactive with human WEE1. The membranes were then stripped and re-probed for β-Actin as a loading control. The nuclear WEE1 and β-Actin bands were quantified using MultiGauge V3.0 software. Two independent experiments were performed and the results from one representative experiment are presented.

We originally hypothesized that the MLL fusion leukemia cell lines, which we expected to have high miR-17-92 levels, would have low WEE1 expression if WEE1 were indeed a target of miR-17-92. Conversely, we predicted that the non-MLL fusion leukemia cell lines, which we expected to have low miR-17-92 levels, would have high WEE1 expression.

Western blot analysis revealed that, as expected, WEE1 is present in the nucleus\textsuperscript{62} (Figure 8A), but not in the cytoplasm (Figure 8C). WEE1 expression from nuclear extracts was quantified relative to β-Actin expression (Figure 8B). Notably, there was no connection between endogenous WEE1 expression and \textit{MLL} rearrangement status. For example, the cell lines with the highest relative expression of WEE1, MonoMac6 and THP-1, were in fact both MLL fusion leukemia cell lines. 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.
Figure 8. 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.
From the Western blot analysis, we can conclude that in the cell lines we tested, endogenous WEE1 expression is dependent on factors in addition to the presence of a MLL fusion protein.

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

We initially hypothesized that high endogenous expression of miR-17-92 in MLL fusion leukemia cell lines would correspond with low endogenous expression of WEE1 in the same cell lines. While we did not see a direct relationship between *MLL* rearrangement status and miR-17-92 expression, we were interested in discerning whether a relationship existed between miR-17-92 expression and WEE1 expression.

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. We observed a negative correlation between the two variables with a coefficient of determination ($R^2$) of 0.6093 (Figure 9A). In general, as miR-17 levels increased, there was a corresponding decrease in WEE1 expression levels. Similarly, we saw a negative correlation between relative expression of miR-19a and relative expression of WEE1 with a coefficient of determination ($R^2$) of 0.5388 (Figure 9B). This result strengthens our conclusion that WEE1 is a valid target of the miR-17-92 cluster.
Figure 9. Negative Correlation between WEE1 Expression and miR-17 or miR-19a Expression

A

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 in shown as well as the equation of the line and the coefficient of determination ($R^2$).
CHAPTER 4
DISCUSSION

Identifying targets of the miR-17-92 cluster is important for expanding our understanding of the complex molecular pathways that regulate gene expression and are dysregulated in leukemia and cancer in general. 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\textsuperscript{43} and Pten\textsuperscript{44}, suppress apoptosis by downregulating E2Fs\textsuperscript{41} and Bim\textsuperscript{42}, and induce angiogenesis in solid tumors by downregulating Tsp1 and CTGF\textsuperscript{45}. Since combined haploinsufficiency of Bim and Pten only partially mimics the oncogenic effects of miR-17-92 overexpression\textsuperscript{44}, it is likely that additional targets contribute to these effects. As such, we were interested in seeking novel targets of the miR-17-92 cluster. In this study, we set out to ascertain which gene transcripts were being targeted for downregulation by the miR-17-92 cluster in MLL fusion leukemias.

We used bioinformatic miRNA target gene prediction algorithms 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. According to MicroCosm Targets, among all the miRNAs predicted to target WEE1, miR-17 had the second highest score, miR-20a had the ninth highest score, and miR-18a had the thirtieth highest score. It is conceivable, therefore,
that multiple miRNAs from the miR-17-92 cluster collectively regulate WEE1 expression.

Our objective for this thesis was to experimentally validate the computationally predicted target WEE1. We hypothesized that overexpression of the miR-17-92 cluster in MLL fusion leukemias leads to downregulation of WEE1, which promotes cell cycle progression and eventually results in leukemia.

Luciferase reporter assays are considered the “gold standard” in validating miRNA gene targets. We performed luciferase reporter assays in which we co-transfected a wild-type or mutant luciferase reporter construct with various combinations of miRNAs from the miR-17-92 cluster. We observed statistically significant increases in luciferase activity when a putative miRNA binding site was mutated in the reporter construct compared to when the site was intact.

From our experiments, it appeared as though miR-17 is the most crucial regulator of WEE1 expression. 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 important. 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. To better decipher the individual contribution of each miRNA of the miR-17-92 cluster to WEE1 regulation, it would be interesting to follow up these studies by expressing each miRNA individually in this assay.

Unexpectedly, we observed small increases in luciferase activity in the presence of the empty vector. This suggests that endogenous miR-17-92 expression in HEK293T
cells might be slightly affecting the assay. It would be interesting to repeat this experiment in a cell line with low endogenous levels of miR-17 and miR-19a, for example, in K-562 cells. These cells would have to be nucleofected with the appropriate constructs because they cannot be transfected easily.

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. A panel of cell lines was selected for this study including MLL fusion leukemia cell lines (MV-4-11, RS4;11, THP-1, MonoMac6), non-MLL fusion leukemia cell lines (K-562, HL-60, U-937), and a control non-leukemia cell line (HEK293T). We isolated RNA from each of the cell lines, prepared cDNA, and then assayed for endogenous miR-17 and miR-19a expression using TaqMan MicroRNA Assays.

Surprisingly, HL-60 and U-937, both of which are non-MLL fusion leukemia cell lines, displayed the highest expression of these two miRNAs. This is not entirely unexpected, though, because HL-60 cells have amplification of the *c-Myc* gene, which has been shown to directly transactivate transcription of the miR-17-92 cluster.
Likewise, U-937 cells are derived from lymphoma cells, and many lymphomas possess amplification of the human locus encoding the miR-17-92 cluster \(^{35}\).

The difference between the relative miR-17-92 expression levels we observed and those that the Chen group observed \(^{47,48}\) may be attributed to inherent differences in expression profiles between cell lines and patient samples. The method of creating new cell lines requires immortalizing primary human cells, a process that can cause changes in gene expression \(^{66}\). Interestingly, TaqMan qPCR data from the Chen group showed that patient samples better followed the expected trend of high miR-17-92 levels in MLL fusion leukemias and low miR-17-92 levels in non-MLL fusion leukemias than cell lines \(^{47,48}\). In fact, they found that miR-17 and miR-20a expression was high in all the leukemia cell lines they tested regardless of MLL rearrangement status \(^{47,48}\). While the Chen group tested ME1, KASUMI-1, and NB4 as their non-MLL fusion leukemia cell lines, we tested K-562, HL-60, and U-937 cells. We did, however, have overlap in the MLL fusion leukemia cell lines tested. Common cell lines included MV-4-11, THP-1, and MonoMac6. The Chen group observed high expression of miR-17 and miR-20a in all three of these cell lines \(^{47,48}\). We also observed fairly high expression of miR-17 and miR-19a in these cell lines, but unlike the Chen group, we did not mean-center our expression data, which makes a direct comparison between the two sets of data difficult.

To further validate WEE1 as a target of the miR-17-92 cluster, we conducted Western blot analysis to assess the relative endogenous expression of WEE1 across a panel of MLL fusion leukemia cell lines (MV-4-11, RS4;11, THP-1, MonoMac6), non-MLL fusion leukemia cell lines (K-562, HL-60, U-937), and a control non-leukemia cell
line (HEK293T). We hypothesized that cell lines with high expression levels of miR-17-92 would express lower levels of WEE1.

We chose to look at WEE1 protein expression using Western blot analysis rather than WEE1 mRNA expression using qRT-PCR because miRNA gene targeting always downregulates protein expression, but only sometimes downregulates mRNA expression. It allowed us to explore whether either of the mechanisms for downregulation, mRNA cleavage or translational repression, occurred. mRNA cleavage takes place when the mRNA has extensive complementarity to the miRNA\textsuperscript{24, 67}. Translational repression occurs when there is insufficient complementarity for mRNA cleavage, but adequate complementarity at multiple sites within the 3’ UTR for translational repression\textsuperscript{24, 67}. 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, we hypothesized that WEE1 mRNA levels remained constant upon miRNA targeting, but WEE1 protein levels decreased.

To enrich for WEE1, which localizes to the nucleus\textsuperscript{62}, we prepared nuclear and cytoplasmic protein extracts. As predicted, we detected WEE1 in the nuclear extracts, but not in the cytoplasmic extracts. 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.

Interestingly, WEE1 electrophoresed slower than expected. While the predicted molecular weight for WEE1 is 72 kDa, product literature for the WEE1 antibody
indicates that the apparent molecular weight on SDS-polyacrylamide gels is typically 95 to 100 kDa. In our Western blot analysis, WEE1 was detected at approximately 150 kDa, which raises the possibility of WEE1 dimerization or the presence of post-translational modifications.

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 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 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, which showed that luciferase activity increased upon co-expression of the miR-17-92 cluster with a reporter plasmid containing mutations in all of the putative miR-17-92 binding sites. 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 the model shown in Figure 10A, 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 (Figure 10B). 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 summary, we have established WEE1 as a novel target of the entire miR-17-92 cluster except for miR-92a. 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. While we did not observe a relationship between miR-17-92 expression and MLL rearrangement status in the cell lines we tested, we demonstrated a specific inverse relationship between endogenous miR-17-92 expression and endogenous WEE1 expression in these cell lines.

The results from this project present several new possibilities for future investigations. It remains to be determined the exact contribution of each miRNA from the miR-17-92 cluster to WEE1 regulation. This can be established by studying each miRNA individually in a luciferase reporter assay. To determine whether miR-17-92 is
Figure 10. Model of the Regulation of WEE1 by the miR-17-92 Cluster

(A) In the absence of the miR-17-92 cluster, WEE1 is translated as normal and WEE1 protein levels remain high. (B) In the presence of high expression of the miR-17-92 cluster in some types of leukemia, miR-17, miR-20a, or miR-18a bind to their target site in the 3’ UTR of WEE1 and miR-19a or miR-19b bind to their target site. Together with the RISC complex, the miR-17-92 cluster inhibits translation of WEE1, which corresponds to a decrease in WEE1 protein levels. Since WEE1 inhibits cell cycle progression, downregulation of WEE1 promotes cell cycle progression and contributes to leukemogenesis.
regulating WEE1 by mRNA cleavage or by translational repression, it would be useful to investigate *WEE1* mRNA levels by qRT-PCR. If mRNA levels are unchanged by changes in miR-17-92 expression, one can deduce that downregulation is occurring via translational repression. If, however, *WEE1* mRNA levels co-vary with WEE1 protein levels, this would suggest that mRNA cleavage is occurring. Additionally, 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 patient samples. Another possible avenue for future research would be to explore whether WEE1 is a target of the miR-17-92 cluster in other types of cancers including solid tumors. A positive finding would suggest that there are broader implications for this regulatory relationship. It also remains to be resolved whether abnormal miRNA expression causes development of the cancer or whether the cancer causes abnormal miRNA expression.

The conclusions from this research suggest that the miR-17-92 cluster and WEE1 are potential therapeutic targets for leukemia. Researchers are already working on developing efficient delivery mechanisms to administer antisense oligonucleotides to inhibit miRNAs such as the oncogenic miR-17-92 cluster. One of the challenges of this approach is that all of the miRNAs of the cluster would have to be inhibited simultaneously due to the functional redundancy of the cluster. Furthermore, developing a mechanism to reintroduce WEE1 into leukemia patients with downregulated WEE1 could prove beneficial in inhibiting uncontrolled cell proliferation. It is my hope that
targeted cancer therapies such as these will continue to make their way from the bench to the bedside.
REFERENCES


VITA

The author, Sonia Olikara, was born in Columbus, Indiana on March 28, 1983 to Cherian and Marina Olikara. She received a Bachelor of Arts degree with a double major in Biochemistry and Spanish from DePauw University in Greencastle, Indiana in May 2004. As an undergraduate student, Sonia was awarded a Science Research Fellowship, where she investigated the molecular phylogenies of rotifers through gene sequencing and began working towards determining the structure of long-chain acyl-CoA dehydrogenase and gamma-glutamylcysteine synthetase.

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