A STUDY OF MICRORNAS ASSOCIATED WITH MULTIPLE MYELOMA
PATHOGENESIS
AND
MICRORNAS/TP53 FEEDBACK CIRCUIT IN HUMAN CANCERS, MULTIPLE
MYELOMA AND Glioblastoma Multiforme

DISSERTATION

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by

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ABSTRACT

MicroRNAs (miRNAs) are single-stranded RNAs of 19-25 nucleotides in length and play a crucial role in regulating gene expression though post-transcriptional gene silencing which leads to mRNA degradation or translational repression. Recently, miRNAs are increasingly implicated in regulating cancer initiation and progression.

Here, we first tried to understand the roles of miRNAs in the pathogenic progress of multiple myeloma (MM), a plasma cell malignancy. In profiling assay of miRNA expression in MM cell lines and CD138+ bone marrow plasma cells (PCs) from subjects with MM, monoclonal gammopathy of undetermined significance (MGUS), and normal donors, we identified overexpression of miR-21, miR-106b-25 cluster, miR-181a and b in MM and MGUS samples with respect to healthy PCs. Furthermore, two miRNAs, miR-19a and 19b, that are part of the miR-17-92 cluster, were shown to down regulate expression of SOCS-1. We also identified p300-CBP-associated factor, a gene involved in p53 regulation, as a bona fide target of the miR-106b-25 cluster, miR-181a and b. Xenograft studies using human MM cell lines treated with miR-19a and b, and miR-181a and b antagonists resulted in significant suppression of tumor growth in nude mice. In summary, we have described a MM miRNA signature, which includes miRNAs that modulate the expression of proteins critical to myeloma pathogenesis.
Next, we investigated the role of miRNAs in the p53 regulatory loop in human cancers, especially, Multiple Myeloma (MM) and Glioblastoma Multiforme (GBM).

In multiple myeloma (MM), we provide evidence that miR-192, 194, and 215, which are downregulated in a subset of newly diagnosed MMs, can be transcriptionally activated by p53 and then modulate MDM2 expression. Furthermore, ectopic re-expression of these miRNAs in MM cells increases the therapeutic action of MDM2 inhibitors in vitro and in vivo by enhancing their p53-activating effects. In addition, miR-192 and 215 target the IGF pathway, preventing enhanced migration of plasma cells into bone marrow.

On the other hand, in human Glioblastoma (GBM) two miRNAs, miR-25 and -32, are identified as p53-repressed miRNAs via p53-dependent, negative regulation of their transcriptional regulators, E2F1 and MYC. In addition, they result in p53 accumulation by directly targeting MDM2 and TSC1, negative regulators of p53 and the mTOR pathway, respectively, leading to inhibition of cellular proliferation through cell cycle arrest. Significantly, overexpression of transfected miR-25 and-32 in GBM cells inhibited growth of the GBM cells in mouse brain in vivo.

Altogether, these results define microRNAs as positive regulators of p53 underscoring their role in tumorigenesis in MM and GBM.
Dedicated to my mother and father
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CHAPTER 1

MINIREVIEW:

GENOMICS OF MICRORNA
In the past decade, there was a distinct advance in biology: the discovery of small RNAs including microRNA (miRNA) and small interfering RNA (siRNA). Since the discovery of RNA interference (RNAi), efforts to identify endogenous small RNAs have led to the discovery of the abundance of miRNAs in diverse multicellular species, including nematodes, fruit flies and humans. In 1993, lin-4 in C.elegans produced a pair of small RNAs that regulate the timing of larval development by translational repression of the lin-14 message without noticeable change in levels of its mRNA (Lee et al, 1993). So far, the number of confidently identified miRNA genes has surpassed 110 in C.elegans, 140 in the fly Drosophila melanogaster, and more than 1500 different miRNAs in humans, which would comprise 1–2% of protein-coding genes in these respective species (Dostie et al, 2003; Grad et al, 2003; Lagos-Quintana et al, 2001; Lai et al, 2003; Lim et al, 2003). Interestingly, nearly all miRNAs are conserved in closely related animals such as human and mouse, or C.elegans and C.briggsae. For instance, at least a third of C. elegans miRNAs have homologs in humans, suggesting that their functions could also be conserved throughout the evolution of animal lineages (Grad et al, 2003). In this review, we summarize the recent advances in miRNA gene identification, biogenesis and expression profiling, with a focus on mammalian miRNAs.

1.1 Genomic distribution and gene structure

Complete definition of miRNA gene structure is ongoing. miRNA genes are dispersed in all chromosomes in humans except for the Y chromosome (Bentwich et al, 2003). Notably, approximately one-third of known miRNAs are clustered in the genome with an organization and expression pattern, implying they are transcribed as
polycistronic primary transcripts. Furthermore, more than half of the clusters contain two or more miRNAs of similar sequence. This genomic arrangement confers a coordinated regulation of miRNAs in the cluster, leading to the combinatorial biological effects via targeting the same gene or different genes in the same pathway. However, although miRNAs in the cluster are coordinately regulated, their expression levels are not the same, suggesting that miRNA are also regulated at the posttranscriptional level (Bartel, 2004).

Initially, it was believed that miRNA genes were mainly located in intergenic regions, but recent studies have shown that a significant portion of mammalian miRNA genes (161 out of 232) were in defined transcription units (TUs), both intronic and exonic; miRNA genes (117 out of 161) were found in the introns in the sense orientation, and of these 117 intronic miRNAs, 90 miRNAs are in the introns of protein-coding genes, whereas 27 miRNAs are in the introns of noncoding RNAs (ncRNAs) (Baskerville et al, 2005; Rodgriguez et al, 2004). In some cases (14 miRNAs), miRNAs are present in either an exon or an intron depending on the alternative splicing pattern (Bracht et al, 2004). So, miRNA genes can be categorized based on their genomic locations: intronic miRNA in protein coding TU; intronic miRNA in noncoding TU; and exonic miRNA in noncoding TU (Figure 1.1). The location of some intronic miRNAs is well conserved more broadly among the diverse species.

miRNAs residing in introns are likely to share their regulatory mechanism and primary transcript with host genes and their expression pattern is closely similar to that of their host gene, as expected for genes sharing the same regulatory promoters (Rodriguez et al 2004). For the remaining miRNAs genes, presumably transcribed from their own promoters, no primary transcripts have been fully understood. By bioinformatic searches
for miRNA-specific promoter elements upstream of miRNA sequences, the characterized miRNA promoters contain general RNA polymerase II (Pol II) transcriptional regulatory elements previously found in protein coding genes (Lee et al, 2004).

Figure 1.1 Genomic organization and structure of miRNA genes (Kim VN et al, 2006)

(a) Intrinsic miRNA in a protein-coding transcriptional unit. (b) Intrinsic miRNAs in a noncoding transcript. (c) The structure of exonic miRNA in noncoding transcripts.
1.2 Biogenesis pathway

The current model for maturation of miRNAs is shown in Figure 1.2.

Transcription of the miRNA gene is mediated by RNA polymerase II (Pol II) (Figure 1.2) and retains mRNA features such as 5’ cap structure and 3’ poly (A) tail (Cai et al, 2004; Lee et al, 2004). Recent analysis of miRNA promoters has also shown that a significant number of miRNA core promoters contain typical Pol II elements such as the TATA box (Kim, 2005; Lee et al, 2004). However, one cannot formally exclude the possibility that a few miRNA genes might be transcribed by another type of RNA polymerase.

Transcription of miRNA genes yields long primary transcripts, pri-miRNAs, which are usually several kilobases long and contain a local hairpin structure (Figure 1.2). This stem-loop structure is recognized and cropped out by the nuclear RNase III Drosha at sites near the base of the primary stem loop to liberate the precursor of miRNA (pre-miRNA) (Lee et al, 2003) (Figure 1.2). Drosha is a large protein of ~160 kDa, which is conserved in animals but not in plants. Drosha forms a large complex of ~650 kDa in human (Filippov et al, 2000; Wu et al, 2000). In this complex, which is known as the Microprocessor complex, Drosha interacts with its cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) encoded protein in humans (also known as Pasha in Drosophila and C. elegans). Because DGCR8 (or Pasha) contains two double-stranded RNA-binding domains (dsRBDS), it is believed to assist Drosha in substrate recognition, although the determinants of its recognition need to be delineated (Denli et al, 2004; Gregory et al, 2004; Han et al, 2004).

Upon being generated by Microprocessor, pre-miRNAs are exported to the cytoplasm of the cell. The nuclear export of pre-miRNA is mediated by one of the
nuclear transport receptors, exportin-5, which a member of the karyopherin family of transport proteins that interact directly with small GTPase Ran (Ras-related Nuclear protein) (Gwizdek et al, 2003; Lund et al, 2004; Yi et al, 2003; Zeng et al, 2004). In the exportin-5 depleted cells, mature miRNAs are reduced but pre-miRNA does not accumulate in the nucleus, suggesting that pre-miRNA could be relatively unstable and might be stabilized through its interaction with exportin-5.

Following cleavage and nucleocytoplasmic export, pre-miRNAs are subsequently processed into ~22-nt miRNA duplexes by the cytoplasmic RNase III Dicer. When Dicer was knocked down or knocked out, pre-miRNAs accumulated, whereas the ~22nt mature miRNAs diminished (Lee et al, 2002). Dicer is a highly conserved protein through almost all eukaryotic organisms such as plants and animals. Similar to Drosha, Dicer associates with a dsRBD containing partner. For instance, Dicer is associated with Ago2 and HIV-1 TAR RNA-binding protein (TRBP) prior to miRNA duplex binding in human cells. Mature miRNAs are incorporated into the effector complexes, which are known as ‘miRNP’, ‘mirgonaute’ or, more generally, ‘miRISC’ (miRNA-containing RNAinduced silencing complex). During RISC assembly, the cleavage products (~22-nt miRNA duplexes) are rapidly converted into single strands. Usually one strand of this short-lived duplex disappears, whereas the other strand remains as a mature miRNA that participates in the process of miRNA-mediated gene silencing.
Figure 1.2 The biogenesis of microRNAs (Esquela-Kerscher A. et al, 2006)
1.3 MicroRNA gene discovery

Recently, considerable effort has been devoted to the identification of novel miRNAs genes. The two general approaches to miRNA gene discovery, bioinformatics and experimental methods, have been applied to the prediction of miRNAs. In general, bioinformatics methods follow the basis of the particular miRNA features used for prediction; all protocols use secondary structure information on the basis of their similarity to the known miRNAs, because stem-loop secondary structure, which should be composed of one arm of the hairpin structure without large internal loops, is an essential characteristic of miRNA (Legendre et al, 2005; Wang et al, 2005). Additionally, the thermodynamic stability of secondary structure is generally considered to distinguish miRNAs from irrelevant genomic hairpins. For example, it has been demonstrated that miRNAs, in contrast to tRNA and rRNAs, have free energies of folding that are significantly lower than those of scraping sequences.

By using RNA-folding algorithms such as MrRscan and miRseeker, approximate hairpin structures can be candidates in non-coding and non-repetitive regions of the genome, and filtered using patterns of phylogenetic conservation between diverse species. Here, known examples of miRNA precursors are used as training references for machine learning algorithms to discriminate between true and false prediction. Computationally predicted candidate miRNAs are validated by experimental procedures such as northern blots, PCR or microarray analysis. In principle, a miRNA can be considered validated when expression of its mature (~22-nt) and the hairpin precursor (~70-nt) is demonstrated. Naturally, bioinformatic predictions have some false-positive rates and can miss species-
specific miRNAs, as evolutionary conservation is widely used as the primary indicator of biological function in many, but not all, of the current approaches.

The traditional experimental approach to miRNA discovery is directional cloning to construct a cDNA library and sequencing (Ambros et al, 2004). The preferred approach to identification of miRNAs is to sequence size-fractionated cDNA libraries. A number of approaches for making cDNA libraries have been developed independently, and these different methods have been successfully applied to identification of most of the currently known miRNAs. All protocols follow the same principle but differ in their detail. Briefly, an RNA sample is separated in a denaturing polyacrylamide gel and the size fraction corresponding to 20-25 nt is isolated. Next, 5’ and 3’ adapters are attached to the RNAs, RT-PCR is carried out and the fragments are cloned into vectors to create a cDNA library. Individual clones are then sequenced and analyzed to determine the genomic origin of candidate miRNAs. The approach successfully identifies endogenous small RNAs, but it is difficult to find miRNAs that are expressed at a low level, in a small number of cells or only under specific cellular conditions. In principle, this limitation can be overcome, at least in part, by deep sequencing of small RNA libraries. More recently, high-throughput sequencing methods, especially 454 sequencing, have become popular for surveying small RNA population.

Both computational and sequencing approaches indicate that there are likely to be many more miRNA, many of which are lineage-specific. Once a small RNA is cloned from a cDNA library, bioinformatics is required to identify its locus in the genome. It may seem a trivial task to determine the genomic location of a 22-nt sequence and to check whether a hairpin structure is conserved in other species. This analysis is
complicated, however, by the fact that hairpin structures are common in eukaryotic genomes and are not a unique feature of miRNAs. Additional care should be taken to distinguish miRNAs from other types of endogenous small RNA and from degradation products of mRNAs.

1.4 Advances in miRNA expression profiling

Expression of most miRNAs is modulated under the control of developmental or tissue-specific signaling, or both. A large number of techniques are available for miRNA detection, including northern blotting, dot blotting, RNase protection assay, primer extension analysis, and quantitative PCR. Large-scale cDNA cloning can also provide information on the relative expression level of miRNAs in diverse samples. However, most of these involve laborious procedures, making it difficult to determine the level of all known miRNAs. Recent advances in quantification of miRNAs expression have easily enabled large-scale expression profiling of miRNAs. Currently, the most commonly used method is based on oligonucleotide miRNA microarrays that are high-throughput tools for assessment of expression levels for hundreds of miRNAs (Babak et al, 2004; Barad et al, 2004; Liu et al, 2004; Schmittgen et al, 2004) (Figure 1.3). Although microarray analysis is a powerful method for miRNA profiling, the small size of miRNAs poses a challenge to the conventional microarray techniques in terms of difficulty to make a single hybridization condition suitable for all miRNAs on the chip. Thus, some of the microarrays employ probes that are complementary to pre-miRNAs rather than mature miRNAs. However, since the maturation process of miRNA is often regulated, the expression level of pre-miRNA does not always synchronize with that of mature miRNA.
Thus, recently developed microarrays detect mature miRNA by applying antisense oligonucleotides that specifically bind to the mature miRNA sequence (Lim et al, 2005).

Figure 1.3 Principles of microarray technology and bead-based flow cytometry used for microRNA profiling (Calin GA et al, 2006)
The most recent innovation in miRNA detection involves the bead-based miRNA profiling technique (Figure 1.3). Each individual bead is marked with fluorescence tags (each color represents a specific miRNA using up to 100 different colors) and coupled to probes that are complementary to specific miRNAs (Shingara et al, 2005). The beads are then analyzed using a flow cytometer capable of measuring bead color. Because hybridization takes place in solution, this method offers higher specificity for closely related miRNAs. This complicated procedure, however, needs to be improved.
CHAPTER 2

MICRORNAS REGULATE CRITICAL GENES ASSOCIATED WITH
MULTIPLE MYELOMA PATHOGENESIS
2.1 ABSTRACT

Progress in understanding the biology of multiple myeloma (MM), a plasma cell malignancy, has been slow. The discovery of microRNAs (miRNAs), a class of small noncoding RNAs targeting multiple mRNAs, has revealed a new level of gene expression regulation. To determine whether miRNAs play a role in the malignant transformation of plasma cells (PCs), we have used both miRNA microarrays and quantitative real time PCR to profile miRNA expression in MM-derived cell lines (n=49) and CD138+ bone marrow PCs from subjects with MM (n=16), monoclonal gammopathy of undetermined significance (MGUS) (n=6), and normal donors (n=6). We identified overexpression of miR-21, miR-106b-25 cluster, miR-181a and b in MM and MGUS samples with respect to healthy PCs. Selective up-regulation of miR-32 and miR-17-92 cluster was identified in MM subjects and cell lines but not in MGUS subjects or healthy PCs. Furthermore, two miRNAs, miR-19a and 19b, that are part of the miR-17-92 cluster, were shown to down regulate expression of SOCS-1, a gene frequently silenced in MM that plays a critical role as inhibitor of IL-6 growth signaling. We also identified p300-CBP-associated factor, a gene involved in p53 regulation, as a bona fide target of the miR106b-25 cluster, miR-181a and b, and miR-32. Xenograft studies using human MM cell lines treated with miR-19a and b, and miR-181a and b antagonists resulted in significant suppression of tumor growth in nude mice. In summary, we have described a MM miRNA signature, which includes miRNAs that modulate the expression of proteins critical to myeloma pathogenesis.
2.2 INTRODUCTION

Multiple myeloma (MM) is a B-cell neoplasm characterized by the accumulation of clonal malignant plasma cells in the bone marrow (Bommert et al, 2006). This cancer can occur de novo or develop from a benign condition called monoclonal gammopathy of undetermined significance (MGUS) at a rate of ~1% per year (Fonseca et al, 2007; Hideshima et al, 2007). MM cells are endowed with a multiplicity of antiapoptotic signaling mechanisms, which account for resistance to current chemotherapy regimens (Oancea et al, 2004). Therapeutic modalities that are effective in MM modulate levels of the proapoptotic and antiapoptotic Bcl-2 family of proteins and of inhibitors of apoptosis, which are primarily regulated by p53 (mutated at low frequency in MM) (Oancea et al, 2004). It is well known that the bone marrow microenvironment plays a prominent role in the biology of MM; adhesion of MM cells to the bone marrow stroma triggers cytokine production, enhances cell proliferation and resistance to chemotherapy by activation of NFκB, phosphatidylinositol 3-kinase PIK/AKT and signal transducer and activator of transcription 3 (STAT-3) pathways through the best characterized MM growth factor, IL-6 (Hideshima et al, 2007; Oancea et al, 2004). Detailed genomic analysis has revealed that MM has complex cytogenetic abnormalities (Drach et al, 1995; Latreille et al, 1982). For example, aneuploidy, assessed by interphase fluorescence in situ hybridization and DNA flow cytometry (Latreille et al, 1982), is observed in ~90% of cases. In addition to chromosome number aberrations, specific cytogenetic abnormalities in MM are typically complex, including reciprocal chromosomal translocations involving the Ig H locus [eg, t (4, 14), t (6, 14), t (14, 16)], chromosome 13 monosomy, loss of the short arm of chromosome 17, and gains or amplifications of the long arm of the chromosome 1.
The presence of RAS family member mutations at codons 12, 13, and 61 of NRAS and KRAS, has been described in ~30 to 35% of MM patients and 45% of MM cell lines (Chung et al, 2007), and more importantly it seems that is the major genetic difference between MM and MGUS. Despite recent advances in oncogenomics and MM cell-stroma interactions, further studies are needed to identify critical players in MM pathogenesis that could be targeted by pharmacological intervention to improve outcomes for this still incurable disease. The advent of new techniques, such as microarray gene expression, including noncoding RNAs, may lead to an improved understanding of MM biology by establishing associations between gene expression changes and MM molecular and clinical features, as shown by us for chronic lymphoid and acute myelogenous leukemia (Calin et al, 2005; Garzon et al, 2008). MicroRNAs (miRNAs) are noncoding RNAs of 19 to 25 nucleotides in length that regulate gene expression by inducing translational inhibition and degradation of their target mRNAs through base pairing to partially or fully complementary sites (Bartel et al, 2004). MiRNAs are involved in critical biological processes, including development, cell differentiation, stress response, apoptosis, and proliferation. Recently, specific miRNA expression patterns have been linked to hematopoiesis and cancer (Croce, 2008; Garzonet al, 2007; Calin and Croce, 2006). Little is known, however, about miRNA expression in MM. A recent study has shown that, in IL-6 dependent MM cell lines, miR-21 transcription is controlled by IL-6 through a STAT-3 mechanism. Moreover, ectopic miR-21 expression is sufficient to sustain growth of IL-6-dependent cell lines in the absence of IL-6 (Loffler et al; 2007). Here, we have used both miRNA microarrays and quantitative RT-PCR to assess the miRNA expression in MM-derived cell lines and
CD138+ bone marrow plasma cells (PCs) from MM subjects, MGUS, and normal donors. Our findings define a miRNA signature related to expression and regulation of proteins associated with malignant transformation of PCs.

2.3 RESULT

2.3.1 A Characteristic miRNA Signature Differentiates MGUS from Healthy PCs.

Current models assume that MM evolves through a multistep transformation process (Fig. 2.1A) (Seidl et al, 2003). To identify specific alterations associated with early pathogenetic events of MM, we profiled five CD138+ PCs from MGUS subjects and four healthy PCs by using our miRNA microarray platform. We first compared MGUS to the healthy counterpart PCs by using the univariate t test within BRB tools (class comparison) (Fig. 2.1B). We found 48 miRNAs significantly deregulated (P=0.05); 41 miRNAs were up-regulated and 7 down-regulated in MGUS with respect to normal CD138+ PCs (Fig. 2.1B). The most up-regulated miRNAs in MGUS were miR-21, which was described by other groups to be up-regulated in MM as well (Loffler et al, 2007), miR-181a, known to have a role in B and T cell differentiation (Chen et al, 2008), and the oncogenic cluster miR-106b-25, in particular miR-93, miR-106b, and miR-25 (see Fig. 2.1B).

2.3.2 miRNA Signatures in MM Patients and Cell Lines

To determine whether miRNAs are deregulated in MM, we analyzed the global miRNA expression in 41 MM-derived cell lines; CD138+ untreated bone marrow PCs from 10 MM, and 4 normal CD138+ PCs using our miRNA microarray (Liu et al, 2008).
First, we compared miRNA expression in primary tumors and cell lines compared to CD138+ healthy controls using univariate t test within by BRB. Our analysis revealed up-regulation of 60 and down-regulation of 36 miRNAs in MM subjects and cell lines compared to CD138+ healthy controls (see Fig. 2.1C). All miRNAs have a fold-change >2 and a P value = 0.01. Because miRNA expression in cell lines could be also deregulated because of prolonged in vitro culture, we analyzed the miRNA expression only in MM subjects versus healthy PCs (Fig. 2.2). We found 37 up- and 37 down-regulated miRNAs in MM subjects with respect to normal PCs with a fold-change 2 and a P value < 0.01 (Figure 2.2). Approximately 90% of the up-regulated miRNAs (34 out of 37) and 30% of the down-regulated miRNAs (10 out of 37) were in common to the combined group of MM subjects and cell lines, thereby validating our approach of combining cell lines and MM subject samples, at least for the analysis of up-regulated miRNAs (see Fig. 2.1C). A Venn diagram in Fig. 2.2 shows the common miRNAs between these two groups of comparison. Similar to the signature observed in MGUS, miR-21 and the miR-106a-92 cluster were found up-regulated in MM subjects and cell lines (see Fig. 2.1C). However, we identified that miR-32 and the cluster miR-17-92 (in particular miR-19a and b) were significantly up-regulated only in MM samples but not in MGUS or healthy PCs (see Fig. 2.1C), indicating a possible role in the malignant transformation from MGUS to MM.

2.3.3 Validation of the miRNA Signatures by q-RTPCR

To validate the microarray results we performed quantitative real time PCR (q-RTPCR) for miR-32, miR-17–5, miR-19a, miR-19b, miR-20a, miR-92, miR-106a (miR-
17-92 cluster), miR-106b, miR-93 and miR-25 (miR-106b-25 cluster), miR-328, and miR-181a and b using an independent set of randomly chosen CD138+ PCs from healthy subjects (n=3), MM patient samples (n=6), and MGUS (n=3), all from different donors, plus a set of MM cell lines (n=15). We confirmed the over-expression of the miR-106b-25 cluster in MGUS and MM samples with respect to the CD138+ healthy PCs (Fig. 2.3A). Although the miR-106b-25 cluster shares a high degree of homology with the miR-17-92 cluster (Fig. 2.1D), and an oncogenic role was reported for both (He et al, 2005; Ventura et al, 2008; Petrocca et al, 2008), we are confident of the specificity of stem-loop q-RT-PCR for the analysis of highly similar miRNAs; a previous report from our laboratory showed exquisite specificity of miR-106b, miR-93, and miR-25 primers (Petrocca et al, 2008). Mature miR-181a was over-expressed in 2 out of 3 MGUS, 6 out of 6 MMs, and 9 out of 15 cell lines with an average on the differential expression shown in Fig. 2.3B. In addition, miR-181b was also over-expressed in MM and MGUS, albeit at a lower degree than miR-181a (see Fig. 2.3B). We further validated the over-expression of the miR-32 and miR-17-92 cluster (Fig. 2.3C–F) in MM patients and cell lines. Consistent with the array data, the two miR-17-92 cluster members, miR-19a and b and miR-32, were highly over-expressed in 6 out of 6 MM PC samples and 15 out of 15 cell lines (see Fig. 3C and F). Principally, we found that miR-19a and b have a fold-change >100 times (see Fig. 3F), although they show very low expression in 1 out of 3 MGUS and almost no expression in 2 out of 3 MGUS and 3 out of 3 healthy PC samples, validating our initial array results and suggesting that these miRNAs are MM-specific.
2.3.4 Several miRNAs Up-Regulated in MM Target p300-CBP-Associated Factor, a Positive Regulator of p53

One of the most up-regulated miRNAs in MGUS and MM patients and cell lines were miR-181a and b, and miR-106b-25, while miR-32 was preferentially up-regulated in MM. Using “in silico” target prediction software [Target Scan (Lewis et al, 2003), Pictar (Krek et al, 2005)], we found that those miRNAs are predicted to target the 3’-UTR of the p300-CBP-associated factor (PCAF) (Fig. 2.4A). PCAF is a histone acetyltransferase involved in the reversible acetylation of various transcriptional regulators, including the tumor suppressor protein p53 (Schiltz et al, 2000). Recently, Linares and colleagues have shown that PCAF possesses an intrinsic ubiquitination activity that is critical for controlling Hdm2 expression levels, and thus p53 (Linares et al, 2007) that is rarely mutated (5–10% of cases) or deleted at diagnosis in MM (Imamura et al, 1994; Stuhmer and Bargou, 2005). To examine whether these miRNAs could regulate PCAF, first we analyzed PCAF expression by q-RT-PCR (Fig. 2.5A) and Western blotting (Fig. 2.5B) in 15 MM cell lines. As control, we used two CD138+ PCs isolated from healthy donors. We found that PCAF expression is almost absent (10-fold less than in control) in 10 out of 15 cell lines, whereas the remaining 5 cell lines displayed very low expression. To investigate whether this gene was deleted at the genomic level we performed whole genome comparative genomic hybridization analysis of all 15 MM cell lines using the Affymetrix SNP 6.0 arrays. However, we did not observe deletion of the PCAF gene (data not shown). Second, we cloned the PCAF 3’ UTR into a luciferase reporter vector and cotransfected with the candidate miRNAs mimics or scrambled oligonucleotides and performed luciferase assays as described in Methods. We found that
miR-181a and b (Fig. 2B), 106b-25 cluster and 32 (Fig. 4 D and B), interact with the PCAF 3’ UTR in vitro. However, this interaction was less significant with miR-92 (Fig. 4C) and no interaction was observed with miR-19a and b (data not shown). Mutation of the predicted miRNA binding sites in the reporter vector abrogated this effect, indicating that these miRNAs directly interact with the PCAF 3’ UTR. To confirm the biological role of these miRNAs in PCAF regulation in MM cells, we validated the in vitro studies by antagonizing endogenous miR-181a, 181b, 25, 93, 106b and 92 using antisense oligos (ASOs) in U266 and JJN3 MM cell lines. In both cell lines the antagomiRs induced accumulation of PCAF protein expression at 72 h after transfection (Fig. 2.4 E and F). By contrast over-expression of the same miRNAs by oligonucleotide transfection reduced PCAF expression in the K562 cell line (Fig. 2.4 G and H). MiR-19a and b did not influence PCAF expression (Fig. 2.4H) and miR-92 had little effect on its expression (see Fig. 2.4F), confirming the luciferase expression data (see Fig. 2.4C). To determine whether the miRNA regulators of PCAF are able to indirectly affect p53 expression, we transfected MM1 cells with anti-miR-181a and b or with all antagomiRs simultaneously (anti-miRs-181s, 93, 106b, 25, 32) exposed the cells to UV (UV) radiation (Fig. 2.4I) and measured the expression of p53 and PCAF by q-RT-PCR (see Fig. 2.4I). Fig. 2.6A shows the re-expression of PCAF protein after 48 h of antagomiRs treatment. The antagonizing activity of transfectant oligos is shown in Fig. 2.6B. After UV treatment, p53 mRNA expression was almost doubled in cells transfected with antisense miR-181a and b, while it increased sixfold after nucleoporation with all antagomiRs simultaneously (see Fig. 2.4I). Furthermore, after transfection of antisense miR-181a and b oligos in MM1s, we treated the cells with a small-molecule MDM2 antagonist nutlin-3a (10 µM) and
measured p53 by Western blotting. We found that the cells transfected with miR-181 ASOs displayed a higher level of p53 protein compared to scrambled and miR-92 ASOs at 9 and 12 h (Fig. 2.4L). Together, these data show that the miR-106b-25 cluster, miR-32, miR-181a and b target PCAF and through this gene, indirectly control p53 activity in myeloma.

2.3.5 miR-19a and miR-19b Target SOCS-1, a Negative Regulator of IL-6R/STAT-3 Pathway

Our findings indicate that miR-19a and b are up-regulated >100-fold in patient samples, >2,000 times in the cell lines (Fig. 2.3F), and are both almost absent in normal PCs and MGUS. These data suggest that both miRNAs contribute to the development of MM. Therefore, we searched for miR-19a and b mRNA targets, which are involved in myeloma pathogenesis, using available target-prediction software [Target Scan, Pictar]. Among >100 predicted targets, SOCS-1 has been implicated in the negative regulation of several cytokine pathways including IL-6, particularly the Jak/STAT pathways (Greenhalgh et al, 2001), and is frequently silenced by methylation in MM (Galm et al, 2002). We therefore hypothesized that the high levels of miR-19s levels in MM samples may play an important role in the constitutive activation of Jak/STAT-3 signaling through down-regulation of its negative regulator SOCS-1. To examine our hypothesis, we first assessed its expression in 15 MM cell lines and two healthy CD138+ PCs and found almost no protein expression in 13 out of 15 cell lines compared to control (Fig. 2.7A). To examine whether SOCS-1 reduced expression levels could be a consequences of up-regulated miR-19a and b in MM cells, we performed Western blot analysis using SOCS-1
antibody after transfection of candidate antagomiRNAs or scrambled oligonucleotides in U266 (which has an active IL-6 autocrine loop) (Schwab et al, 1991) and JJN3 MM cell lines that display reduced SOCS-1 expression (Fig. 2.7D). Moreover, miR-19a and miR-19b mimics inhibited the expression of a reporter vector containing SOCS-1 3’UTR, while mutation of the predicted miRNA-binding site abrogated this effect (Fig. 2.7 B and C). As shown in Fig. 2.7 C and D, there was significant up-regulation of SOCS-1 protein in U266 and JJN3 cells transfected with antisense oligonucleotide for miR-19a and b but not with scrambled oligonucleotides. Furthermore, constitutive STAT-3 phosphorylation in U266 cells was markedly decreased at 72 h after transfection by anti-miR-19s but not by scrambled (Fig. 2.7E). The ASOs activity at 72 h was detected by q-RT-PCR (Fig. 2.7F). These studies suggest a role of miR-19s in the IL-6 antiapoptotic signal in the pathogenesis and malignant growth of MM.

2.3.6 miR-17-92 Cluster Target BIM in MM Cells

Because the miR-17-92 cluster has been shown to target the proapoptotic gene, BIM (Ventura et al, 2008; Petrocca et al, 2008), we examined whether BIM expression is modulated by miR-17-92 in MM cells. U266 cells were transfected with miR-19s ASOs and BIM expression was evaluated using immunoblot. We found a significant increase of BIM protein levels at 48 h after treatment with anti-miR-19s compared to scrambled oligonucleotides (Fig. 2.8). Together, these results have supported previous published data (Ventura et al, 2008; Petrocca et al, 2008) that show BIM as a direct target of miR-17-92 and suggest a possible mechanism through which over-expression of miR-17-92 contributes to the antiapoptotic signals in MM.
2.3.7 Ectopic Repression of miR-19s and miR-181a and b in MM Cell Lines Leads to Significant Suppression of Tumor Growth in Nude Mice

To explore the in vivo relevance of our observations, we examined the tumorigenicity of U266 and JJN3 cells in athymic nu/nu mice after silencing of the endogenous miR-19s and miR-181a and b. Two independent experiments, each using 16 mice for each cell line, were conducted. U266 or JJN3 cells were transfected with ASOs or scrambled oligonucleotides in vitro. We confirmed transfection efficiency (80% for U266 and JJN3) using BLOCK-IT Fluorescent Oligo (Invitrogen). Twenty-four hours later 3 x 10^7 viable cells for each group, re-suspended in 100 µl of BD matrigel matrix, were injected into the right flank of nude mice. The relative expression levels of the endogenous miR-19s and miR-181a and b after ASO transfection was assessed by q-RT-PCR to confirm the down-regulation of these miRNAs in both MM cell lines (data not shown). We found that after 2 weeks of cell inoculation, 70% of mice injected with MM cells transfected with scrambled oligonucleotide developed measurable tumors. By contrast, mice transplanted with cells expressing antagomiRs showed significant inhibition of tumor growth compared with controls (P < 0.01) (Fig. 2.9 A-B). Both cell lines treated with anti-miR-19s showed tumor volumes >10-fold reduced and tumors treated with antago miR-181s were three times smaller in JJN3 cells and 10 times smaller in U266 cells, P<0.02 and P<0.01, respectively. Importantly, complete tumor suppression was observed in two mice injected with U266 cells treated with anti-miR-19s. The average tumor volume after 4 weeks for U266 cells was 308.5 mm^3 and for JJN3 cells was 225 mm^3. Only 50% of mice injected with MM cells transfected with the antago miR-19s and miR-181s developed measurable tumors at 4 weeks. The average tumor
volume for U266/anti-miR-19s was 19.5 mm$^3$ and for U266/anti-miR-181s was 14 mm$^3$.

Similar results were observed with JJN3 MM cells, with average tumor volumes of 25 mm$^3$ and 80 mm$^3$ for anti-miR-19s and anti-miR-181s, respectively (see Fig. 2.9 A and B). Taken together, these results strongly suggested an oncogenic role of these miRNAs in MM and we speculate that the stronger effect of anti-miR-19s is related to the IL-6 dependence of U266.

2.4 DISCUSSION

Over the past few years several studies have illustrated the biological relevance of miRNAs for the differentiation of normal hematopoietic cells and the contribution of deregulated miRNA expression in malignant counterparts (Volinia et al, 2006; Garzon et al, 2008). Our study has provided a unique comprehensive global miRNA expression profiling of MM, MGUS and contrasted these expression patterns with that of normal PCs. The similar miRNA expression pattern observed in MM cell lines and in primary newly newly diagnosed MMs supports the research design of this study. In addition, previous MM microarray studies have combined MM cell lines with primary patient samples, validating our strategy (Zhan et al, 2002). We sought to identify a miRNA signature that could be associated with a MM multistep transformation process from normal PCs via MGUS to clinically overt myeloma, conscious of limitations because of the low number of MGUS and primary tumor samples. In MGUS patients, we identified up-regulated miRNAs with oncogenic function, such as the miR-21 and the miR-106b-25 cluster. MiR-21 is up-regulated in many solid and hematological tumors (Croce, 2008; Imamura et al, 1994). The ectopic expression of miR-21 in glioblastoma cells blocks apoptosis
(Chan et al, 2005), while silencing its expression in several cancer cells inhibits cell growth and leads to increased apoptotic cell death by unblocking the expression of its targets: tumor suppressor genes like phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4) (Meng et al, 2007). Petrocca and colleagues (Petrocca et al, 2008), have shown that the miR-106b-25 cluster plays a role in gastric cancer tumorigenesis by targeting the proapoptotic BIM and p21. Thus, these two miRNAs may contribute to earlier steps in plasma cell transformation by blocking apoptosis, promoting PC survival, and predisposing to secondary genetic abnormalities that will ultimately result in a full blown malignancy. In MM (including MM cell lines and primary tumors versus normal PCs) we identified a signature comprised of multiple up-regulated miRNAs, including among others miR-32, miR-21, miR-17-92, miR-106-25, and miR-181a and b. While miR-106-25, miR-181a and b, and miR-21 were up-regulated also in MGUS patients with respect to normal PCs, miR-32 and the miR-17-92 cluster were highly expressed only in MM patients, suggesting that these miRNAs are MM-specific. Besides RAS mutations, no other genetic abnormality has been found to differentiate MGUS from MM (Hideshima et al, 2007). Therefore, miR-32 and the miR-17-92 cluster may represent MM-specific genetic changes. However, this will require further independent validation because we used a small number of patient samples and normal plasma cells. Similar to the miR-106-25 cluster, the oncogenic role of the miR-17-92 cluster in B cell lymphoma is well known and several known proapoptotic genes, including PTEN, E2F1, and Bcl2l11/ BIM are confirmed as targets of miR-17-92 (Novotny et al, 2007; O’Donnell et al, 2005). Recently, Ventura and colleagues (Ventura et al, 2008) have shown that the miR-17-92 cluster is also essential for B cell
development and that the absence of miR 17-92 leads to increased levels of the proapoptotic protein BIM and inhibits B cell development at the pro-B to pre-B transition. However, given the nearly identical sequences, it is very likely that miR-106b-25 and miR-17-92 clusters cooperate in exerting similar, if not identical, functions as in targeting BIM. Our study provided important functional insights about miRNAs deregulated in MM. We have confirmed that the proapoptotic BIM is a target of the miR-17-92 cluster in MM cells. Therefore, miR-17-92, along with miR-21, blocks apoptosis and promotes cell survival. On the other hand, miR-106b-25, miR-181a, and miR-32 but not miR17-92 cluster (specifically miR-19s and miR-92), target PCAF, a p53 positive regulator. We speculate, consistent with the low frequency of p53 mutations in MM, that down-regulation of PCAF by the miR-106b-25 cluster, miR-181s, and miR-32, keeps p53 at low level or partially inactivated by controlling its stability through Hdm2 (Linares et al, 2007) and working as a histone acetyltransferase (Schiltz et al, 2000). We have also described the specific role of miR-19s on the STAT-3/IL-6R negative regulator SOCS-1. In fact, IL-6 pathways in MM are among the best characterized survival pathways participating in PC transformation and oncogenesis through STAT-3, impacting apoptosis regulators such as the Bcl-2 family members (Bommert et al, 2006). Our findings demonstrate that the up-regulation of miR-19s in MM could contribute to SOCS-1 down-regulation and IL-6 activation at later stages in MM pathogenesis. The role of miR-19s and miR-181s in MM cells as oncomiRNAs was confirmed by in vivo studies. Our data demonstrate significant tumor regression of transplanted tumors after treatment with miR-19s and 181s antagomiRs. These data may suggest that miRNAs could have a therapeutic potential in antagonizing the growth of transformed PCs. In conclusion, we
reported distinctive miRNA signatures in MM and MGUS, characterized by over-expression of miRNAs with known oncogenic activity. Our data provided insights into the miRNA function in MM by establishing links with the regulation of critical pathways in MM by miRNAs, including apoptosis, survival, and proliferation. These results indicate an additional level of control by this class of regulatory molecules in the multistep process associated with malignant transformation of PCs.

2.5 MATERIAL AND METHODS

2.5.1 RNA Extraction and miRNA Microarray Experiments

RNA extraction and miRNA microchip experiments were performed as described in detail elsewhere. The miRNA microarray is based on a one-channel system. Five micrograms of total RNA was used for hybridization on the Ohio State University custom miRNA microarray chips (OSU-CCC version 3.0), which contains ~1,100 miRNA probes, including 345 human and 249 mouse miRNA genes, spotted in duplicate.

2.5.2 RT-PCR

The single tube TaqMan miRNA assays were used to detect and quantify mature miRNAs as previously described (10) using ABI Prism 7900HT sequence detection systems (Applied Biosystems). Normalization was performed with RNU6B. Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method.

2.5.3 ASOs and Mimics Transfection Experiments.
Cells were transfected by using nucleoporation (Amaza) kit V (for JJN3 and MM1s cell lines) and kit C (for U266 cell line) using 100 nM miRNA precursors (Ambion), or 100 nM LNA miRNA antisense oligonucleotides (Ambion). Protein lysates and total RNA were collected at the time indicated. miRNA processing and expression were verified by Northern blot and stem-loop q-RT-PCR. We confirmed transfection efficiency (~80% for U266 and JJN3 and 50% for MM1s) using BLOCK-IT Fluorescent Oligo (Invitrogen) for all of the cell lines. Untreated cells transfected with negative control oligonucleotides were used as a calibrator.

2.5.4 Cell Collection and Total RNA Purification

Samples included PCs from 16 newly diagnosed cases of MM, 6 patients with MGUS, and 6 healthy donors (normal PCs). Written informed consent was obtained in keeping with institutional policies. PC isolation from mononuclear cell fraction was performed by immunomagnetic bead selection with monoclonal mouse anti-human CD138 antibodies using the AutoMACs automated separation system (Miltenyi-Biotec Three of the six healthy PCs were obtained from ALLCELLS, and the purity of PCs was assessed by FACScan Analysis and was more than 80%. MM cell lines [courtesy of Dr M. Kuehl (National Cancer Institute, MD), Dr. Joshua Epstein (Little Rock, AR), Dr. S. Rosen (Chicago, IL), Dr. M. Gramatzki (Kiel, Germany)] and an EBV-transformed B-lymphoblastoid cell line (ARH-77, ARK, UCLA-1) were grown as recommended (American Type Culture Collection). Total RNA was isolated with TRIzol extraction reagent (Invitrogen).
2.5.5 Luciferase Reporter Vectors

PCAF and SOCS1 3’UTR containing predicted microRNA binding site were amplified by PCR from genomic DNA (293T/17cells) and inserted into pGL3 control vector (Promega) by using an XbaI site immediately downstream from the stop codon of firefly luciferase. Deletion of the first six nucleotides of each complementary seed-region complementary site was inserted in mutant constructs using quick-change site-directed mutagenesis kit (Stratagene), according to the manufacturer’s protocol. Primer sequences are available upon request.

2.5.6 Luciferase Assays

QBI293 and Meg01 cells were cotransfected in six-well plates with 1 g of pGL3 firefly luciferase reporter vector (see luciferase reporter vector method), 0.1 g of the phRL-SV40 control vector (Promega), and 100-nM miRNA precursors (Ambion) using Lipofectamine 2000 (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by using the Dual Luciferase Assay (Promega) 24 h after transfection. Each reporter plasmid was transfected at least twice (on different days) and each sample was assayed in triplicate.

2.5.7 Xenograft Model

Studies were performed under an Institutional Animal Care and Use Committee (IACUC) approved protocol. Eight week-old male athymic nu/nu mice (Charles River Laboratories) were maintained in accordance with IACUC procedures and guidelines. A mixture of 30 x 106 U266 cells or JJN3 transfected cells were suspended in 0.10 ml of
extracellular matrix gel (BD Biosciences) and the mixture was injected s.c. into the right and left flanks. Serial measurements of xenograft growth were performed, and tumor volume was estimated using the formula $4/3 \pi (L*W*H/8)$.

2.5.8 Western Blotting

Immunoblot analyses were performed as described (10), using: rabbit polyclonal antiSOCS-1 (Abcam); rabbit polyclonal antisera against GADPH, Bim, Stat-3, P-Stat-3 (Tyr-705) (Cell Signaling); rabbit polyclonal PCAF, and monoclonal p53 (Santa Cruz). Protein levels were normalized relative to ACTIN or GAPDH level, detected with appropriate antisera (Santa Cruz Biotechnology).

2.5.9 UV and Nutlin3a Treatment

MM1s and U266 MM cell lines were transfected with miR-181s, miR-106b-25, miR-32, and miR-19s ASOs as described above, and at 24 h after transfection U266 cells were treated with 10µM Nutlin3a (Cayman Chemical Company) and MM1S were treated with 6 J/m² UV (Ultra LUM. Inc.) and harvested at the time described in Results.

2.5.10 Data Analysis

Microarray images were analyzed by using GenePix Pro 6.0. Average values of the replicate spots of each miRNA were background-subtracted and subject to further analysis. MiRNAs were retained when present in at least 50% of samples and when at least 50% of the miRNA had a fold-change of more than 1.5 from the gene median. Absent calls were thresholded to 4.5 in log2 scale before normalization and statistical
analysis. This level is the average minimum intensity level detected above background in miRNA chip experiments. Quantile normalization was implemented using the Bioconductor package/function. Differentially expressed microRNAs were identified by using the univariate t test within the BRB tools version 3.5.0 set with a significant univariately at alpha level equal to 0.01. This tool is designed to analyze data using the parametric test t/F tests, and random variance t/F tests. The criteria for inclusion of a gene in the gene list is either a P value less than a specified threshold value, or specified limits on the number of false discoveries or proportion of false discoveries. The latter are controlled by use of multivariate permutation test.
Figure 2.1 MM and MGUS express a distinct spectrum of miRNA in comparison to normal CD138+PCs (A) Schematic drawing showing the multistep molecular process of PC transformation. (B) Representative list of the miRNAs significantly deregulated in MGUS versus normal PCs. The asterisks indicate the specific associated cluster. (C) Representative list of the common deregulated miRNAs in the comparison classes MM patient versus normal PCs and PCs MM versus normal PCs. (D) Three families of miRNA precursors can be identified.
Figure 2.2 Common miRNAs expression. A Venn Diagram showing the common miRNAs (C) between the two class of comparisons, MM PCs versus healthy PCs (A) and PCs from MM patients versus healthy PCs (B).
Figure 2.3 Validation of microarray data in MM patients, MGUS and MM cell lines versus CD138+ PCs healthy by q-RT-PCR

Average miR-93, miR-25 and miR-106b (A), miR-181a and miR-181b (B), miR-32 (C), miR-17–5 and miR-20a (D), miR-92 and miR-106a (E), and miR-19a and miR-19b (F) expression in CD138+ PCs from healthy donors (n=3), from MGUS (n=3), from MM patients (n=6), and MM cell lines (n=15) measured by q-RT-PCR. Bars represent relative fold-changes between MM PCs, MGUS, and healthy PCs. The miRNA expression between the different groups was compared after normalization with RNU6B.
Figure 2.4 miR-181s, 106b-25 cluster, 32 target PCAF

(A) miRNAs predicted to interact with PCAF gene in several consensus binding sites at its 3'-UTR, according to “in silico” target Target Scan prediction software. (B–D) Luciferase assay showing decreased luciferase activity in cells cotransfected with pGL3-PCAF-3_UTR and miR-181s (B), miR-32/miR-25/miR-92 (C), and miR-93/miR-106b (D) oligonucleotides. Deletion of six bases in three putative miR-181s, miR-106b/miR-93, and miR-32/miR-25/miR-92 binding sites, complementary to miRNAs seed regions, abrogates this effect (MUT). Bars indicate firefly luciferase activity normalized to Renilla luciferase activity. Each reporter plasmid was transfected at least twice (on different days) and each sample was assayed in triplicate. (E, F) Immunoblot analyses showing PCAF and GADPH expression after transfection with miR-181s and miR-106b/25 cluster ASOs in U266 cells (E) and with miR-181s, miR-32 and miR-92 ASOs in JNJ3 cells (F), or mimics in K562 cells (G, H). (I) Real-time RT-PCR analyses for p53 and PCAF expression in MM1s cells transfected with miR-181a and miR-106b/25 together ASOs (pool) or with scrambled oligonucleotide at 48 h after transfection and after 4 h of UV treatment. The PCR products for both genes were normalized to GADPH and ACTIN expression. The bar-graph represents the mean values observed in four separate studies_ SE. (L) Immunoblot analysis showing p53 protein expression after 48 h of miR-181a, miR-181b, miR-92 and scrambled ASOs transfection in MM1 cells after 9 h and overnight incubation with 10 _M nutlin-3a; GADPH was internal loading control. Densitometry based on GADPH levels shows increased level of p53 in presence of miR-181a and miR-181b ASOs in MM1s cell.
Figure 2.5 PCAF expression in MM cell lines. (A) Real-time RT-PCR analyses for PCAF expression in 15 MM cell lines and 1 healthy CD138+ PC sample.

The PCR product was normalized to GADPH and ACTIN expression and each point was repeated in quadruplicate; differences were significant (P<0.001). (B) Immunoblot analysis of 15 MM cell lines showing expression of PCAF normalized for ACTIN expression.
Figure 2.6 Validation of ASOs transfection

(A) Immunoblot analysis showing PCAF protein expression in MM1s cells at 48 h after miR-181a/b, miR-106b-25 cluster, miR-32 (pool) or miR-181a/b or scrambled ASOs transfection in MM1s cells; GADPH was internal loading control. (B) Stem-loop q-RT-PCR to validate the expression of endogenous miR-181a/b, miR-106b-25 cluster, miR-32 at 48 h after transfection of MM1s cells with antagonizing oligonucleotides, after normalization with U6.
Figure 2.7 miR-19s target SOCS-1 in MM cell lines

(A) Immunoblot analysis with antisera against SOCS-1 and GADPH in 15 MM cell lines and two CD138+PCs from healthy donors (control). (B) Predicted highly conserved consensus binding site in human, mouse, rat, and dog for miR-19s on the 3’UTR of SOCS-1. (C) Relative luciferase activity in MEG01 cells transiently cotransfected with luciferase reporter vector containing the 3’ UTR of SOCS-1 and miR-19s or scrambled oligonucleotides. Deletion of the six bases in the putative miR-19s binding site, complementary to miRNA seed region, abrogates this effect (MUT). Bars indicate firefly luciferase activity normalized to Renilla luciferase activity. Each reporter plasmid was transfected at least twice (on different days) and each sample was assayed in triplicate. (D) Western blot showing SOCS-1 protein in whole cell lysates from U266 and JJN3 cells at 48 h after transfection with scrambled oligonucleotides or miR-19a, miR-19b, or both ASOs. GADPH was used as loading control. Densitometry based on GADPH levels shows increased level of SOCS-1 in presence of miR-19a or miR-19b or together ASOs in U266 and JJN3 cells. (E) Western blot showing that miR-19s modulate expression of activated STAT-3 in U266 cells in vitro. Cells were transfected with anti-miR-19s or negative control (Scr) miRNA inhibitors in vitro, and cell lysates were obtained after 72 h. Densitometry based on STAT-3 levels shows decreased level of p-STAT-3 in presence of miR-19s. (F) Stem-loop q-RT-PCR to validate the expression of endogenous miR-19a and miR-19b, at 72 h after transfection of U266 cells with antagonizing oligonucleotides, after normalization with RNU6B
Figure 2.8 miR-17–92 cluster targets Bim. Immunoblot analyses showing BIM-EL, BIM-L, and GADPH expression at 48 h after transfection with miR-19a or miR-19bor together ASOs in U266 cells. Densitometry based on GADPH levels shows increased level of BIM-EL and BIM L in the presence of miR-19b and miR-19a and b together ASOs in U266 cells.
Figure 2.9 Antagonizing miR-19s and miR-181s expression in MM cell lines resulted in significant tumor suppression in nude mice. U266 and JJN3 cells (30_106 cells) were injected subcutaneously into the flanks of nude mice with 100 _l of matrigel solution 24 h after transfection with miR-19s and miR-181s ASOs or scrambled oligonucleotide. Mice were killed on day 35 and tumor volumes were calculated. (A) Time course of tumor growth of U266 cell line. (B) Time course of tumor growth of JJN3 cell line; tumors treated with miR-19s and miR-181s ASOs were significantly smaller than tumors of scrambled groups for both cell lines. Scale bar indicates 10 mm.
CHAPTER 3

DOWNREGULATION OF P53-INDUCIBLE MICRORNAS 192, 194, AND 215 IMPAIRS THE P53/MDM2 AUTOREGULATORY LOOP IN MULTIPLE MYELOMA DEVELOPMENT
3.1 ABSTRACT

In multiple myeloma (MM), an incurable B cell neoplasm, mutation or deletion of p53 is rarely detected at diagnosis. Using small-molecule inhibitors of MDM2, we provide evidence that miR-192, 194, and 215, which are downregulated in a subset of newly diagnosed MMs, can be transcriptionally activated by p53 and then modulate MDM2 expression. Furthermore, ectopic re-expression of these miRNAs in MM cells increases the therapeutic action of MDM2 inhibitors in vitro and in vivo by enhancing their p53-activating effects. In addition, miR-192 and 215 target the IGF pathway, preventing enhanced migration of plasma cells into bone marrow. The results suggest that these miRNAs are positive regulators of p53 and that their downregulation plays a key role in MM development.

3.2 INTRODUCTION

The tumor suppressor p53 is frequently inactivated by mutations or deletions in cancer. p53 acts as a potent transcription factor and can be activated in response to diverse stresses, leading to induction of cell-cycle arrest, apoptosis, or senescence (Junttila and Evan, 2009; Xue et al, 2007). Although regulation of the p53 pathway is not fully understood at the molecular level, it has been well established that activated p53 suppresses cancer progression, underlining why cancer cells have developed multiple mechanisms to disable p53 function (Danovi et al, 2004; Ventura et al, 2007). In human tumors that retain wildtype (WT) p53 (Junttila and Evan, 2009; Lane, 2001) p53 can be antagonized by murine double minute 2 (MDM2), a negative regulator of p53 that is also overpriced in many human tumors, offering a therapeutic strategy (Dickens et al, 2009).
Brown et al, 2009). It has been reported that inhibiting MDM2 expression can reactivate p53 in cancer cells, leading to their demise (Dickens et al, 2009; Saha and Chang, 2010). TP53 mutation is rarely detected at diagnosis in many hematological cancers such as multiple myeloma (MM), acute myeloid leukemia, chronic lymphocytic leukemia, and Hodgkin’s disease (HD). Thus, numerous reports have shown that therapeutic induction of p53 might be particularly suitable for the treatment of hematological malignancies (Saha and Chang, 2010). Among them, multiple myeloma (MM) is a currently incurable plasma cell proliferative disorder that results in considerable morbidity and mortality (Kuehl and Bergsagel, 2002; Fonseca et al, 2009). MM develops from a benign condition called monoclonal gammopathy of undetermined significance (MGUS) (Weiss et al, 2009). Individuals with MGUS often remain stable for years and do not require treatment. However, for unknown reasons, this benign condition can evolve into MM at a rate of ~1% per year, with some MMs developing after many years (Kuehl and Bergsagel, 2002; Fonseca et al, 2009). In MGUS and in the majority of newly diagnosed MM cases TP53 is WT (Kuehl and Bergsagel, 2002; Chng et al, 2007) and the protein is rarely detectable (Stuhmer and Bargou, 2006). Interestingly, in MM cells, expression of p53 protein levels can be rescued by antagonizing MDM2. Several reports have focused on the p53-mediated apoptotic pathway, upon endogenous p53 protein re-expression by the small-molecule MDM2 antagonists (Nutlins) and target genes which may be involved in p53-dependent apoptosis in MM cells have been identified (Stuhmer and Bargou, 2006). MicroRNAs are an abundant class of short, non-protein coding RNAs that mediate the regulation of target genes posttranscriptionally and that have emerged as master regulators in diverse physiologic and pathologic processes (Bartel, 2004), and
oncogenesis (Croce, 2008). Recently, microRNAs (miRNAs) have been reported to be directly transactivated by p53 (He et al, 2007). miRNAs have also been shown to target p53 and/or components of p53 regulatory pathways, thereby directly and/or indirectly affecting its activities (Park et al, 2009; Zhang et al, 2009). We previously published the global miRNA-expression profiles of MM and MGUS and contrasted these profiles with those of normal plasma cells (PCs) (Pichiorri et al, 2008). The findings defined a miRNA signature related to expression and regulation of proteins associated with malignant transformation of PCs, such as p53 (Pichiorri et al, 2008). We have now examined the regulation and functional roles of miRNAs in MM development using small-molecule inhibitors of MDM2.

3.3 RESULTS

3.3.1 Identification of p53-Regulated miRNAs in MM

To determine if miRNAs are regulated by p53 in MM cells, we performed custom microarray analysis with an expanded set of probes capable of assaying the expression of more than 500 human miRNAs. Two approaches to compare the effect of p53 level on miRNA expression were used. We first assessed a specific signature associated with the presence of WT TP53 in MM cell lines as shown in Figure 3.1A and Table 3.1 available online. Six MM cell lines were used in the analyses: MM1s, NCIH929, and KMS28BM which retain and express WT TP53; RPMI-8226, U266 with mutant TP53; and JJN3 that does not express TP53 mRNA. Western blot analysis of these cells shows their p53 and MDM2 steady-state protein levels, respectively (Figure 3.2A). Genomic and cDNA sequence analyses confirmed the presence of WT TP53 cells
in association with higher MDM2 mRNA expression (Figure 3.2B). Several differentially expressed miRNAs were identified (Table 3.1). Some of these miRNAs, such as those of miR-34 family, have been found to be associated with p53 status in other human malignancies (Hermeking, 2010). In the second approach, we performed miRNA microarray analysis in MM1s cells treated with or without Nutlin-3a (10 mM), a small-molecule inhibitor of MDM2 (Figure 1B). In response to Nutlin-3a treatment, we identified expression of distinct miRNAs associated with p53 activation (Figure 3.1B; Table 3.2). Only two miRNAs were upregulated in both analyses: miR-34a and miR-194 (Figures 3.1A-B; Tables 3.1 and 3.2). These results not only confirm upregulation of miR-34a as a function of wild type (WT) p53 status (He et al., 2007) but also point to strong upregulation of miR-194 by p53 (Tables 3.1 and 3.2; Figures 3.1A-B). The significant upregulation of miR-192 and miR-215 after p53 re-expression through Nutlin-3a treatment is especially interesting because they are located, together with miR-194, in two related microRNA clusters, the miR-194-2-192 cluster at 11q13.1 and the miR-215-194-1 cluster at 1q41.1 (Table 3.2), and have the same seed sequence. Genomic locations of these two clusters have been reported to be important for MM (Fonseca et al, 2009). miR-194 also has the same mature sequence regardless its expression cluster and miRNAs of the same cluster are usually expressed together (Garofalo et al, 2009; Ventura et al, 2008).

3.3.2 p53 Induces the Expression of miR-192, 194, and 215

To confirm the microarray data, we first tested by q-RT-PCR for the presence of miR-34a, miR-194, and its cluster associate, miR-192 and 215, in WT TP53 compared
with Mut TP53 cells (Figure 3.2C). WT TP53 cells retained higher expression of miR-34a, miR-194, and miR-192 (Figure 3.2C), but did not show expression of miR-215, suggesting that the 11q13.1 miR-194- 2-192 cluster is associated with WT TP53 status in MM cells. To determine the kinetics of p53 activation, we treated MM1s cells with 10 mM Nutlin-3a for different duration of time. p53 was barely detectable by immunoblotting at 6 hr but increased after 12, 18, and 24 hr of treatment and remain constant at 30–36 hr (Figure 3.1C). The induction of p53 was also associated with MDM2 accumulation, p21 expression, and c-MYC downregulation after 12 hr of treatment (Figure 3.1C). The expression of p21-encoding CDKN1A, a p53 target gene, was also assessed by RT-PCR (Figure 3.1D). By northern blot and qRT-PCR analysis, we also studied the kinetics of miRNA activation during p53 upmodulation in MM1s cells. The kinetics of miR-34a, miR-194, miR-192, and miR-215 expression (Figure 3.1E) were directly correlated with p53 protein upregulation and p21 activation (Figures 3.1C-D), while for miR-15 and miR-29a/b the dynamics of expression appeared more related to downregulation of their repressor c-MYC as expected (Chang et al, 2008; Figures 3.2D-E; Figure 3.1D), than to p53 activation. To confirm the responsiveness of these miRNAs to p53, cell lines with varying TP53 status were treated with Nutlin-3a or vehicle (DMSO), followed by qRT-PCR to monitor miRNA levels upon p53 activation (Figures 3.2F–K). Induction of miR-34a, miR-192, miR-215, and miR-194 was detected only in the cell lines treated with Nutlin-3a and harboring WT TP53 (p < 0.001, Figure 3.2). Next, we analyzed induction of these miRNAs, by Nutlin-3a in freshly isolated CD-138+ PCs (Figure 3.2L), from eight bone marrow aspirates of MM patients. Two samples (Pt-1 and Pt-2) exhibited TP53 deletion by FISH analysis, while 6 (Pt-3 to Pt-8) retained TP53
genes (Table 3.3). We detected induction of p53, miR-34a, 192, 194, 215 after 12 hr of Nutlin-3a treatment (Figures 3.2M-O) in TP53 WT samples, in association with different levels of CDKN1A mRNA activation (Figure 3.2O). Furthermore, to determine if these miRNAs are relevant in MM pathogenesis, we analyzed the expression of miR-194, 192, and 215 in a panel of CD138+ PCs obtained from newly diagnosed MM patients (n = 33), MGUS (n = 14) patients and normal donors (n = 4) (Table 3.3) by qRT-PCR (Figures 3.1F-H). Through Kruskal-Wallis analysis, we found that these clusters of miRNAs are consistently downregulated in MM samples (p < 0.001) compared with MGUS samples.

3.3.3 Identification of the p53 Core Element in the pri-miR-192-194-2 Promoter at 11q13.1

To determine if p53 is directly involved in the transcriptional regulation of miR-194-2-192 and miR-215-194-1 clusters, we analyzed the cluster promoter regions. The upstream genomic region close to the transcription start site (TSS) (+1) (Hino et al, 2008) of pri-miR-194-2-192 contains several highly conserved regions among human, mouse, rat, and dog sequences (from ~162 to +21 with respect to the TSS). To identify the promoter region responsive to p53 re-expression, we constructed reporter plasmids carrying various genomic sequences around the TSS of the pri-miR-194-2-192 cluster and subjected them to luciferase assay (Figure 3.3A). Bioinformatics search identified a previously reported high score p53 consensus site between ~900/~912 bp (Sinha et al, 2008; Song et al, 2008); however, this site was not functional for p53 activation since luciferase reporter constructs excluding this region retained full activity (P3-P7) (Figure 3.3A). The region from ~245 to +186 bp (P7) had promoter activity comparable to that of
the longest regions in MM cells after forced expression of p53 (Figure 3.3A), but regions from ~125 to +186 bp (P8 and ~912 to ~245 bp (P10) did not. We identified a p53-responsive element between ~245 and ~125 bp (Figure 3.3B) because the construct excluding this region was not affected by p53 expression (Figure 3.3A). Since conserved regions in a given gene promoter are expected to contain regulatory elements, we focused on the highly conserved region controlling luciferase activity, the region between ~161 and ~135 bp. Luciferase assay using a construct mutated for each C and G contained in the two decamers of the hypothetical El-Deiry consensus sequence revealed that this unpredicted and previously unpublished region is critical for p53 transcriptional activation of the pri-miR-194-2-192 cluster (Figure 3.3B). Indeed, we found that endogenous p53 directly interacts with the core element of the pri-miR-194-2 promoter in MM1s cells, as demonstrated by CHIP assay after 12 hr of Nutlin-3a treatment (Figure 3.3C). As a positive control, we used the p53 consensus site on the miR-34a promoter, while a nonspecific sequence served as negative control (Figure 3.3C). Ectopic expression of p53 activated the promoter of both members of the pri-miR-194-2-192 cluster in MM1s cells (Figure 3.3D). MDM2 siRNA after p53 re-expression in MM1s cells led to higher relative luciferase activity and thus confirmed its dependence on p53 re-activation. Taken together, these data suggest that p53 is a key transcriptional activator of pri-miR-194-2 through directly binding to the core promoter element (Figure 3.3D).

We also attempted to identify the promoter and primary transcript of miR-215-194-1 on chromosome 1q41.1, but could not identify the primary transcript initiation point by 30 and 50 RACE and PCR amplification of the putative transcribed sequences (ESTs);
however, we confirmed the previously published consensus site for p53 by CHIP analysis (Figure 3.4).

3.3.4 miR-192, 194, and 215 Affect p53-Dependent MM Cell Growth
To examine the relevance of p53-mediated regulation of miR-192, 194, and 215 in MM, we first tested whether reintroduction of these miRNAs affected the biology of MM cells. Previous studies (Georges et al., 2008; Braun et al., 2008) showed that such reintroduction induced expression of p21 in different cancer cell lines which carried WT TP53, with a consistent G0/G1 arrest and p53 protein expression. The molecular mechanism of p53 re-expression remained elusive. To confirm this effect in our model, miR-192, miR-194, and miR-215 were introduced into transfection in WT TP53-cell lines (MM1s, NCI-H929, and KMS28BM), as well as cells with mutated TP53 (RPMI-8226), followed by detection of TP53 and mRNAs of target genes CDKN1A and MDM2, by RT-PCR analysis (Figure 3.5). We found consistent re-expression of CDKN1A in TP53 WT cells (Figure 3.5A) after transfection, but did not detect any increase in TP53 mRNA (Figure 3.5B). Using MTS assay, we observed significant growth arrest in the cells transfected with miR-192, 215 and a less significant arrest with miR-194 in MM cells carrying WT TP53 (Figures 3.6A–C), as compared with scrambled sequences. In contrast, we did not detect this effect in RPMI-8226 cells (Figure 3.6D) expressing mutant TP53. Next, we determined if p53-responsive miRNAs interfere with the clonogenic survival of MM cells. MM cells were lentivirus-transduced with miR-192, miR-215, miR-194 and miR-34a, miR-192, and 215 in WT p53 cells suppressed colony formation to an extent comparable to miR-34a, which was used as an internal control. Of note, miR-194 was less effective
than miR-215 and miR-192. These miRNAs did not suppress colony formation in RPMI-8226 (Figures 3. 6E and 3.6F) or U266 cells (F.P., S.-S.S., C.M.C, unpublished data), while miR-34a did exhibit colony suppression in these mutant TP53 cells, confirming its p53-independent apoptotic action (Hermeking, 2010).

To further explore the p53-dependent mechanism(s) of miR-192, 215, and 194 interferences with cell growth and colony formation, we used flow cytometry to determine if their expression affects progression through the cell cycle. We noted that in the two WT TP53 cell lines with high expression of MDM2 mRNA, MM1s and NCI-H929 (Figure 3.2B), the p53-responsive miRNAs induced a consistent G0/G1 arrest. This effect was observed in ~30% of scrambled-transfected cells versus ~60% of the cells transfected with miR-192 and 215 and ~45% for miR-194 (Figures 3.6G-H). By contrast in KMS-28BM cells, retaining WT TP53 but expressing lower levels of MDM2 mRNA (Figure 3.2B), we detected increases of sub-G1 fractions (indicative of cell death) in cells transfected with miR-192 (~25% sub-G1), 215 (30%), and 194 (12%) at 48 hr after transfection, compared with ~3% in control cells transfected with the Scr sequence (Figure 3.6I). At 48 hr after transfection, we also detected increased caspase-3 activity (Figure 3.6J). The differential effect of the miRNAs on TP53 WT cells carrying lower MDM2 mRNA basal expression (Figure 3.2B) led us to analyze MDM2 levels after miRNA transfection (Figure 3.5C). MDM2 mRNA, but not protein, was detected after MM cells transfection. Because MDM2 protein is rapidly autoubiquitinated and degraded through the proteasome pathway (Marine and Lozano, 2010); p53 induction is necessary for its detection in MM cells (Stuhmer and Bargou, 2006; Ooi et al, 2009). Only in one (Mut TP53 RPMI-8226) of six MM cell lines analyzed was MDM2 protein detected
without p53 activation (Figures 3.2A and 3.2G). We also noted that MDM2 mRNA was downregulated after ectopic expression of these miRNAs, mostly in WT TP53 cells, but to some extent also in Mut TP53 cells (RPMI-8226) (Figure 3.5C). These data were confirmed at the protein level in RPMI-8226 cells where we observed ~20% downregulation of MDM2 protein at 72 hr after miRNA transfection (Figure 3.5D). The results indicate that ectopic expression of miR-192, 215, and 194 in WT TP53 cells inhibits cell growth and enhances apoptosis, effects that could be related to MDM2 regulation in MM cells.

3.3.5 Human MDM2 Is a Direct Target of miR-192, 194, and 215

The data thus far demonstrate that the biological functions of miR-192, 215, and 194 in MM cells is p53 dependent. After introduction of these miRNAs the TP53 mRNA level did not change in MM cells but higher CDKN1A and lower MDM2 mRNA levels were observed (Figure 3.5). Both genes, MDM2 and CDKN1A, are direct targets of p53 but their expression in this case was not preceded by TP53 transcription (Figure 3.5B). Thus, we hypothesized that miR-192, 194 and 215 could target the expression of MDM2. To further examine the effects of these miRNAs on MDM2 protein expression in WT TP53 MM cells, we analyzed the consequences of ectopic expression of miR-192, miR-194, and miR-215 at 72 hr after transfection and 12 hr of nongenotoxic activation of p53 by Nutlin-3a (10 mM). Increased expression of these miRs upon transfection was confirmed by qRT-PCR (Figure 3.7A), and the effects on p53, MDM2, and p21 levels were analyzed by western blot (Figure 3.8A). Overexpression of miR-192, 194, and miR-215 significantly increased the level of p53 and p21 at 12 hr after Nutlin-3a treatment.
compared with Scr-transfected cells (p < 0.001), as shown by densitometric analysis in Figures 3.8B and 3.8C. Expression of MDM2 protein was dramatically decreased in both cell lines (Figures 3.8A–C). Conversely, knockdown by 2’-O-me-anti-miR-192-194 and 215 (pool) after 12 hr of Nutlin-3a treatment, as confirmed by qRT-PCR (Figure 3.7B) in TP53 WT cell lines, increased the level of MDM2 protein (p < 0.01), while p21 and p53 protein levels were attenuated (p < 0.01) (Figure 3.8B), as confirmed by densitometry (Figure 3.8E). We also confirmed that MDM2 mRNA levels were strongly reduced in the miR-192-, 194-, and 215- transfected cells at 6 and 12 hr of Nutlin-3a treatment in both cell lines (Figures 3.8C). These results indicate that miR-192, 194, and 215 induce the degradation of MDM2 mRNA, confirming that they regulate both, protein and RNA levels. We next tested whether MDM2 is a direct target of these miRNAs by performing a bioinformatics search (Target Scan [Lewis et al, 2003]; Pictar Pictar [Krek et al, 2005]) but were unable to identify the 3’UTR of MDM2 as a target. Because the 3’ UTR of MDM2 is not well conserved across species, we decided to use the RNA22 target prediction program (Miranda et al, 2006) which does not need validated targets for training, and neither requires nor relies on cross-species conservation. RNA22 predicted two miRNA-responsive elements (MREs) for miR-192/215 and two MREs for miR-194 in the 3’UTR of human MDM2 (HDM2) (Figures 3.8D; Figures 3.7C–3.7G). To verify that HDM2 is a direct target of miR-192, miR-194, and miR-215, HDM2 3’UTR containing all MREs (~4K), was cloned into pGL3 basic construct downstream of the luciferase open reading frame (Figure 3.8D). This reporter construct was used to transfect MM1s cells which express the endogenous miRs following upmodulated p53 expression. Increased expression of these miRs upon transfection significantly
diminished luciferase expression (Figure 3.8D). We subsequently screened the predicted MREs on the 3’UTR of HDM2 mRNA, using luciferase assays with four different constructs carrying the MREs for miR-192/215 and miR-194 (Figures 3.7H–K). We observed that expression of each specific MRE reporter construct was specifically downregulated upon transfection of each individual miRNA. Conversely, when we performed luciferase assays using a plasmid harboring the binding sites inactivated by site-directed mutagenesis, we observed a consistent reduction in the inhibitory effects (Figures 3.7H–K). We also analyzed the expression of MDM2 mRNA in a panel of CD138+ PCs obtained from MM patients, MGUS patients, and normal donors by RT-PCR (Figure 3.8E). Through Kruskal-Wallis analysis, we found that MDM2 mRNA is significantly upregulated in MM samples (p < 0.001) compared with MGUS samples and normal PCs (Figure 3.8E). Using nonparametric test analysis, we found a significant inverse correlation between miR-192 expression and MDM2 mRNA in MM samples (Sperman r = -0.698, p < 0.0001, n = 33) (Figure 3.8F).

3.3.6 miR-192, 194, and 215 Re-expression Enhances Sensitivity of WT TP53 MM Cells to Nongenotoxic Activation of p53 In Vitro and In Vivo

To determine if re-expression of the miRNAs could enhance sensitivity of WT TP53 MM cells to nongenotoxic activation of p53, we tested MI-219, a highly selective, orally active small-molecule inhibitor of the MDM2-p53 interaction (Shangary et al, 2008). We first examined whether MI-219 induces p53, MDM2, p21, and Puma upregulation in MM1s cells after miR-192, miR-194, and miR-215 transfection. In cells with forced expression of miR-192 and 215, p53 became detectable even in untreated
cells (Figures 3.9A-B) (p < 0.05), confirming previously published data in other cell lines (Georges et al, 2008; Braun et al, 2008). p53 re-expression and subsequent p21 and Puma upregulation was observed in these cells and in miR-194-transfected cells and is clearly visible following 24 hr of 2.5 mM MI-219 treatment (Figure 3.9A). In control cells (Scr), the treatment was ineffective (Figure 3.9A). Densitometric analysis of p53 and MDM2 protein levels was performed when cells were treated for 24 hr with 2.5, 5, and 10 mM MI-219 (Figure 3.9B). Confirming our previous data, we observed higher p53 accumulation (R2-fold increase, p < 0.001) and dramatic MDM2 downregulation (R3-fold decrease, p < 0.001) in miRNA-transfected cells (Figure 3.9B). These opposing changes in MDM2 and p53 expression levels correlated with higher activation of p53 downstream targets, p21 and Puma (Figure 3.9A). Furthermore, MI-219 induced higher caspase-3 activation in the presence of miR-192, miR-194, and miR-215 (p < 0.001) (Figure 3.9C). Next, we examined whether activation of p53 by MI-219 leads to apoptosis in MM cells. Indeed, treatment with MI-219 induced apoptosis as revealed by Annexin V staining (Figure 3.9D). In cells transfected with a pool of miRNAs, MI-219 effectively (p < 0.0002) induced apoptosis at 2.5 mM (27% ± 3%) and 5 mM (32% ± 3%) while the scrambled control did not. This effect was less significant when using MI-219 at 10 mM (30% ± 5%), though it was enhanced when treatment was combined with miRNAs (55% ± 5%) (Figure 3.9D). Increased concentration of MI-219 did not increase the apoptotic rate of scrambled-transfected cells but caused nonspecific toxicity (data not shown). Because previous studies have shown that MI-219 achieved excellent oral availability, we investigated if, in mouse xenograft models, the combined action of miRNAs and oral MI-219 could suppress tumorigenicity of MM cells. Viable MM1s
Gfp+/Luc+ cells (8 x 10^6) were injected subcutaneously into the right flank of 40 nude mice. At 3 weeks after injection a group of 32 mice with comparable tumor size were selected and randomly divided into four groups for four independent experiments, using eight mice for each combined treatment (Figure 3.9E). Specifically, we used the combination of oral treatment with 200 mg/kg MI-219 or vehicle control (VE) once a day for 14 days plus direct tumor injection of double-strand RNA scrambled sequence (Scr) or a pool of pre-miR-192, 194, and 215 (miRs). Whereas the VE-Scr-treated tumors increased 2-fold in volume in 2 weeks (from 5390 ± 993 mm^3 to 13,500 ± 3200 mm^3 [p < 0.0001]), MI-219/Scr-treated tumors remained static in volume (5390 ± 993 mm^3 to 5400 ± 1200 mm^3) (Figure 3.9E). By contrast, mice treated with VE-miRs showed ~1.5-fold reduction in tumor size (from 5390 ± 993 mm^3 to 3700 ± 950 mm^3 [p < 0.01]). The most effective combination was MI-219 plus miRs, where mice showed 5-fold reduced tumor volumes (from 5390 ± 993 mm^3 to 2100 ± 560 mm^3 [p < 0.01]) and >93% reduction when compared with VE/Scr treatment (Figure 3.9E). These findings demonstrate proof-of-concept for in vivo therapy of MM using combined miRNAs and an MDM2 pharmacological inhibitor.

3.3.7 miR-192 and miR-215, by Antagonizing MDM2 Downregulation, Target IGF-1 and IGF1-R

Since our data demonstrate that miR-192, miR-194, and miR-215 target MDM2, we sought to determine if MDM2 substrates could also be affected. IGF-1R is a known target of MDM2 ubiquitin ligase function (Girmita et al., 2003; Froment et al., 2008). Therefore, by targeting MDM2, miR-192, 194, and 215 may indirectly influence the
expression of IGF-1R. In MM cells, IGF-1R and its ligand, IGF-1, are key factors in regulation of PC migration into the bone marrow (Qiang et al, 2004; Tai et al, 2003). We noted that in WT TP53 MM cells, p53 re-expression was strongly associated with downregulation of IGF-1R and IGF-1 (Figures 3.10A-B) compared with mutant TP53 MM cells (Figures 3.10C-D). We sought to determine the effect of miRNAs on IGF-1R and IGF-1 expression through targeting MDM2. We found that in the presence of miR-192, 215 but not miR-194, IGF-1R and IGF-1 protein levels decreased, as determined by western blot analysis (Figure 3.11A). Furthermore, inhibition of endogenous miR-192/215, using antisense oligonucleotides, combined with 24 hr of Nutlin-3a treatments increased both IGF-1R and IGF-1 levels in MM1s cells (Figure 3.11B). This effect was not seen with miR-194. To determine if the regulation of IGF-1 does affect IGF1R in MM cells, we silenced IGF-1 and observed upregulation of IGF-1R protein levels at 48 and 72 hr posttreatment (Figure 3.11C). This effect was clearly different from that observed following miRNA transfection (Figure 3.11A). We next tested whether miR-192 and 215 target IGF-1R and IGF-1 directly by generating luciferase reporters containing their 30 UTRs. Using Targetscan (Lewis et al, 2003), Pictar (Krek et al, 2005), and RNA22 (Miranda et al, 2006) searches, we identified several MREs for miR-192 and miR-215 but not for miR-194 in the 30UTR of IGF-1R and IGF-1R mRNAs (Figures 3.11D-E). Luciferase activity dropped 40%–50% when these constructs were cotransfected into MM1s cells with miR-192, 215 compared with miR-194 and Scr (Figures 3.11D-E; Figures 3.10E–I). To determine if these miRNAs could regulate IGF-1 and IGF-1R expression, we transfected miR-192 and 215 (pool) into PCs of nine patients. We observed a significant decrease in IGF-1R and IGF-1 protein expression (Figures
3.11F and 11G). Transfection efficiency was confirmed using RNA Fluorescent Oligo (Figure 3.11H). These results indicate that miR-192 and miR-215 directly target IGF-1R and IGF-1 in MM.

3.3.8 miR-192 and 215 Block MM Migration and Invasion In Vitro and In Vivo

Given the known role of IGF-I and IGF-1R as antiapoptotic factors and in MM migration through endothelial barriers and bone marrow stroma (Qiang et al, 2004; Tai et al, 2003), we sought to determine if miR-192 and 215 interfere with the chemotactic function of IGF-I and block migration and invasion of MM cells. We first determined that miR-192 and 215 actions on the IGF-1 axis in MM affect both normal TP53 (MM1s) and mutant (RPMI-8226) cell lines (Figure 3.12A) and that the downregulation of both proteins critically affects S6 and AKT phosphorylation in these cells. Next, we found that ectopic expression of miR-192 and 215 in NCI-H929 and RPMI-8226 IGF-1-treated cells was associated with significant decrease in cell adhesion (Figures 3.13A-B), migration, and tissue invasion compared with Scr control. To this end, we used an intraepithelial trans-well migration assay with IGF-1 at various concentrations as attractant and two bone marrow-derived stromal cells, HS-5 (fibroblast-like) and HS-27A (epithelial-like) as cell layer. As shown in Figure 3.12B and Figure 3.13C, IGF-1 (50 ng/ml) stimulated migration of MM cells, MM1s, and RPMI-8226. To further examine the role of miRNA-192, 215, and 194 in MM cells, we investigated the effect of these miRNAs on migration in vivo, using a previously described homing model (Roccaro et al, 2009). Nine NOD-SCID mice (for each group) were intravenously injected with 8 x 10^6 of pre-miRNA-192-, 194-, and 215- or Scr probe-transfected GFP+/Luc+MM1S cells. One week later, mice
were injected intravenously every week for 4 weeks with an individual miRNA or Scr dissolved in PBS (10 mg for each mouse). After 5 weeks, the homed and proliferated tumors were markedly suppressed in miRNA-treated mice compared with Scr-transfected MM cells (p < 0.01) (Figure 3.12C). At 5 weeks postinjection, we first noted reduced tumor progression, by bioluminescence imaging (Figure 3.12D). Mice injected with Scr-transfected MM1s in addition to serial intravenous injection with Scr showed significant tumor growth, but tumor burden was significantly reduced in mice injected with pre-miR-194 and was nearly nonexistent in mice injected with either pre-miR192 or pre-miR-215 (Figures 3.12C-D). In addition, through FACS analysis using human CD-138+ antibody, we analyzed bone marrow engraftment of these cells in injected NOD-SCID mice. We confirmed that Scr-treated mice showed bone marrow engraftment of ~25% ± 15% of MM1s cells versus 4% ± 2% for miR-192 and 2% ± 2% for miR-215 animals (Figure 3.12E). The in vivo action of miR-194 was less effective, 12% ± 3% of bone marrow engraftment MM1s cells compared with miR-192 and 215 but still higher than the Scr control (Figure 3.12E). These data indicate that miR-192, 215 but also miR-194 have therapeutic potential not only by affecting proliferation rates in MM cells but also by affecting the homing and migration ability of MM cells.

3.3.9 The Promoter Region of the miR-194-2-192 Cluster is Hypermethylated in MM Cell Lines

During Nutlin-3a treatment of primary CD-138+ PCs from MMs without TP53 deletion, we noted that p21 activation, as well as re-expression of the three miRNAs, was consistent but not uniform in all samples analyzed. Possibly mechanisms other than
alterations to the TP53 gene could influence the sensitivity to MDM2 molecular inhibitors. In seeking an explanation for the lack of expression of these miRNAs in MMs, we noted that the genes for these miRNAs are located in chromosomal regions in MM that are normally characterized by chromosome gain and translocations rather than deletions (Fonseca et al, 2009). We then explored the methylation status in the promoter of the miR-194-2-192 cluster. By combined bisulfite restriction analysis (COBRA), we detected hypermethylation of the promoter region of this cluster (Region R) (Figure 3.14A) in MM cell lines (Figure 3.14B). Furthermore, treatment of MM cell lines with a demethylation agent (Azacytidine) increased the expression of these miRNAs in WT TP53 MM cell lines (Figure 3.14C).

3.4 DISCUSSION

Complex cytogenetic abnormalities and numeric chromosomal aberrations occur in virtually all multiple myeloma, and in most, if not all, cases of MGUS (Kuehl and Bergsagel, 2002). Paradoxically, mutations and/or deletion of TP53 occur in only a small percentage of intramedullary MMs and not at all in MGUS (Chng et al, 2007). Several reports (Teoh et al, 1997; Quesnel et al, 1994) and our data (Figure 3.8E) suggest that MDM2 overexpression in MMs, but not its gene amplification (Quesnel et al, 1994), could be responsible for p53 inactivation in cells retaining functional p53 pathways. This supports the idea that induction of p53 in this setting might be a suitable treatment for MM. There have been a few reports concerning microRNA deregulation in MM and although not all reported findings were in agreement, they confirmed that such deregulation is important in MM pathogenesis (Pichierri et al, 2008; Roccaro et al, 2009;
Lionetti et al, 2009; Gutie’rrez et al, 2010). Here, we studied the role of miRNAs in the p53 apoptotic pathway upon nongenotoxic activation of p53 in MM cells, using small molecular inhibitors of MDM2 (Nutlin-3a, MI-219). Upon p53 activation, we identified two related microRNA clusters located in regions considered important for MM (miR-194-2-192 at 11q13.1 and miR-194-1-215 at 1q41.1) (Fonseca et al, 2009). Furthermore, the knowledge that miRNAs coming from the same cluster can reinforce their action on the same cellular pathways (Garofalo et al, 2009; Ventura et al, 2008) led us to study the molecular mechanisms associated with activation of the p53 pathway by these miRNAs. Through characterization of the miR-194-2-192 cluster promoter region and definition of a noncanonical p53 consensus site, we have shown that these miRNAs are direct p53 targets. In patient samples, the expression of these miRNAs changed during transition from normal PC, via MGUS to intramedullary MM; these miRNAs were significantly downregulated in a cohort of newly diagnosed MMs versus MGUS; miR-192, 215, and 194 enhanced colony suppression, cell-cycle arrest, or apoptosis in a p53-dependent manner. We also noted, as in the case of KMS28BM cells, that their biological action could be associated with the MDM2 status in MM cells. The short half-life of MDM2 protein (Marine et al, 2010) and difficulties in analyzing its protein expression in MM cells without p53 activation led to the demonstration that the effect of these miRNAs on MDM2 was clearly detectable after treating WT TP53 MM cells with combined Nutlin-3a and ectopic expression of the miRNAs. In fact, we observed that in treated cells with enforced expression of these miRNAs MDM2 was dramatically downregulated at protein and mRNA levels and this downregulation was inversely associated with higher p53 expression and p21 activation (Figures 3.8A-C). Luciferase assays using plasmids
harboring the MDM2 3’UTR sequence strongly confirmed that MDM2 is the direct target of these miRNAs. In a subset of newly diagnosed MMs, elevated levels of MDM2 mRNA were inversely associated with miR-192 expression. We proved, in vivo and in vitro, that the combination of these miRNAs with p53 pharmacological activator (MI-219), leading to MDM2 downregulation and subsequent p53, p21, and Puma upregulation, could be a successful therapeutic strategy. In fact, it produced anti-tumor results that could not be achieved solely by increasing the drug concentration. We also found that miR-192 and miR-215 expression, by overriding MDM2 ubiquitination of IGF-1R (Girnita et al., 2003; Froment et al., 2008), directly targets the IGF-1 axis in MM cells, controlling mobility and invasive properties of MM cells in vitro and in vivo. We propose a model in which these miRNAs are regulators of the autoregulatory loop, increasing the window of time between p53 apoptotic action and p53 degradation by MDM2; and at the same time targeting the IGF axis, antagonizing the MDM2 ubiquitin ligase function on IGF-1R (Figure 3.15).

The hypermethylation status in the promoter of the miR-194-2-192 cluster in MM cell lines could support the hypothesis that the transition from MGUS to MM is favored by clonal selection of cells with aberrant promoter methylation of the miR-194-2-192 cluster. This could be associated with a decreasing ability of p53 to downmodulate MDM2 expression thus tipping the regulatory balance in favor of MDM2 in MM cells. This proposed model (illustrated in Figure 3.14D) will require further investigation.

Of note, monoallelic deletion of TP53 in MM, which often seems to occur without mutation on the other allele, is associated with an extremely poor prognosis (Chng et al., 2007). This supports the idea that a 2-fold decrease in TP53 gene content is associated
with tumor progression, which supports the hypothesis that a partial lack of expression of these miRNAs in MMs could create a p53 imbalance with direct biological consequences.

In summary, our results have defined a mechanism of p53 regulation through miRNAs acting on MDM2 expression, providing the basis for the development of miRNA-targeted therapies for MM, as illustrated in Figure 15.

3.5 MATERIALS AND METHOD

3.5.1 CD-138 + PCs purification, MM cell lines collection and growth conditions

Plasma cells, CD 138+ cells were purified from total marrow cells of patients by Human Whole Blood CD138+ Selection Kit (Cat#18387, Stem Cell Technologies) as per the manufacturer’s instructions. Yield and purity of CD138+ cells was evaluated by flow cytometry using anti-CD138 antibody (Becton Dickinson). Primary cells that were used for in vitro experiments were cultured in RPMI-1640 (Sigma) supplemented with 15% fetal calf serum and kept in culture for 24 h before specific treatment. MM cell lines (MM1s, NCI-H929, KMS28, RPMI-8226, U266 and JJN3) [courtesy of Dr M. Kuehl (National Cancer Institute, MD) were cultured in RPMI-8226 (Sigma) and 10% fetal bovine serum (Cat#019K8420, Sigma). Human bone marrow stromal cell lines HS-27A and HS-5 were purchased from American Type Culture Collection (Chantilly, VA) and cultured in RPMI 1640 containing heat-inactivated 5% fetal bovine serum (FBS).

3.5.2 Transfection method for primary cells and MM cell lines

Specifically for primary cells 1 X10^6 CD-138+ PCs were resuspended in 100 µl of V solution and 100 nM miRNA precursors (Ambion) was used for the transfection reaction.
For MM cell lines 5 $\times 10^6$ cells were re-suspended in 100 µl of nucleofector solution V/C and 100 nM of miRNAs precursor or or 100 nM LNA miRNA antisense oligonucleotides (Ambion) was used for each transfection point. Protein lysates and total RNA were collected at the time indicated. miRNA processing and expression were verified by northern blot and stem-loop qRT-PCR. We confirmed transfection efficiency using BLOCK-IT Fluorescent Oligo (Invitrogen) for all the cell lines. Untreated cells transfected with negative control oligonucleotides were used as a calibrator.

3.5.3 RNA-DNA-Protein extraction from primary cells

Total RNA-DNA-Protein from primary CD-138+ PCs was extracted using RNA/DNA/Protein purification kit from NORGEN (cat# 23500, Thorold, ON, Canada) following the manufacture’s instructions. Briefly 350 µl of lysis solution was added to 1 $\times 10^6$ CD-138+ PCs pellet. The cells were lysed by vortexing and 200 µl of 95% ethanol was added to the lysate. The entire lysate volume was loaded to the provided columns. After several column washes the RNA was eluted using 35 µl of RNA Elution Solution. The same column was then washed with 500 µl of gDNA and the genomic DNA using 40 µl of gDNA Elution buffer. For the protein extraction the flowthrough from the RNA binding step was applied following the manufacture’s instructions onto the provided column, washed and eluted using 100ul of the provided buffer.

3.5.4 RNA extraction from MM cell lines

Total RNA from MM cell lines (RPMI-8226, U266; JJN3; NCI-H929; MM1s; KM28BM) was extracted using TRIzol Reagent Invitrogen (Cat# 15596-018) following
the manufacture’s instruction. Specifically the pellet obtained from $5 \times 10^6$ cells was lysed 1 ml of TRIzol solution. At the end of the extraction the isolated RNA was dissolved in 35 µl in RNase-free water and incubated for 10 min at 55°C.

3.5.5 Microarray experiments

The total RNA from MM cells used for microarray analysis was isolated with TRiz extraction reagent (Invitrogen) as previously described. miRNA microchip experiments were performed as described in detail elsewhere (Liu et al., 2004). The miRNA microarray is based on a one-channel system. Five micrograms of total RNA was used for hybridization on the OSU custom miRNA microarray chips (OSU-CCC version 3.0), which contains 1,100 miRNA probes, including 345 human and 249 mouse miRNA genes, spotted in duplicates. The data were analyzed by microarray images by using GenePix Pro 6.0. Average value of the replicate spots of each miRNA was background-subtracted and subjected to further analysis. MiRNAs were retained when present in at least 50% of samples and when at least 50% of the miRNA had fold change of >1.5 from the gene median.

3.5.6 Data Analysis for microarray experiments

Microarray images were analyzed by using GenePix Pro 6.0. Average values of the replicate spots of each miRNA were background-subtracted and subject to further analysis. MiRNAs were retained when present in at least 50% of samples and when at least 50% of the miRNA had fold change of more than 1.5 from the gene median. Absent calls were thresholded to 4.5 in log2 scale before normalization and statistical analysis.
This level is the average minimum intensity level detected above background in miRNA chips experiments. Quantiles normalization was implemented using the Bioconductor package/function. Differentially expressed microRNAs were identified by using the univariate t test within the BRB tools version 3.5.0 set with a significant univariately at alpha level equal to 0.01. This tool is designed to analyze data using the parametric test t/F tests, and random variance t/F tests. The criteria for inclusion of a gene in the gene list is either p-value less than a specified threshold value, or specified limits on the number of false discoveries or proportion of false discoveries. The later are controlled by use of multivariate permutation test.

3.5.7 q-RT-PCR

The single tube TaqMan miRNA assays from Applied Biosystems (miR-192#000491; miR-215#000518; miR-194 #000493; miR-34a#000425; miR-15a#000389; miR-29a#002112; miR-29b#000413) were used to detect and quantify mature miRNAs as previously described (Garofalo et al., 2009) using ABI Prism 7900HT sequence detection systems (Applied Biosystems). Normalization was performed with RNU44 (Applied Biosystems Assay #00194) or RNU48 (Applied Biosystems Assay #001006). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative $C_t$ method.

3.5.8 Western Blot Analysis

Samples were extracted in 15 mM Tris_Cl, pH 7.5/120 mM NaCl/25 mM KCl/2 mM EGTA/0.1 mM DTT/0.5% Triton X-100/10 mg/ml leupeptin/0.5 mM PMSF. Total
protein (35 µg) from each sample was separated on a 4–20% Tris-HCl Criterion precast gel Bio-Rad (cat# 345-0032, Hercules, CA) and transferred to a poly(vinylidene difluoride) filter (Millipore). The filter was blocked in 5% nonfat dry milk, incubated with the specific antibody, washed, and probed with secondary antibody IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and developed with enhanced chemiluminescence (Amersham Pharmacia). Immunoblot analyses were performed using the following antibodies: p53 (sc-53394, Santa Cruz Biotechnology), MDM2 (sc-965, Santa Cruz Biotechnology), phospho-MDM2 (Cat#3521, Cell Signaling), c-MYC (cs-40, Santa Cruz Biotechnology), IGF-1 (sc-9013, Santa Cruz Biotechnology), IGF-1R(Cat#3027, Cell Signaling), total-Akt (Cat#9272, Cell signaling), phospho-Akt (Cat#4060, Cell Signaling), total-S6 (Cat#2217, Cell Signaling), phospho-S6 (Cat#2211, Cell Signaling), p21 (sc-817, Santa Cruz Biotechnology), α-PUMA (Cat#4976, Cell Signaling), GAPDH (Cat#2118, Cell Signaling). Filters were reprobed with enzyme-conjugated antibodies to GFP and β-actin (Santa Cruz Biotechnology).

3.5.9 Nutlin3a and MI-219 treatment

MM cells (from MM patients or from cell lines) non-transfected or transfected with pre- or ASOs miR-192, miR-215, miR-194 and Scr sequence as described above, were treated with MDM2 inhibitor (Nutlin3a and MI-219). Fresh CD138+ primary PCs isolated from new diagnosed MM patients as previously described were maintained in culture for 24 hr and then treated for 24 hr with 10 µM Nutlin-3a (Cayman Chemical Company) or vehicle (DMSO). For transfected cells at 24 hrs after transfection MM cells were treated with 10 µM Nutlin-3a (Cayman Chemical Company) or DMSO vehicle only at different time
points. MM1S cells were also treated with MI-219 solution (10% PEG400 / 3% Cremophor EL / 87% 1X PBS) or only vehicle (10% PEG400 / 3% Cremophor EL / 87% 1X PBS) at different concentration (2.5, 5 and 10 µM) for 24 hr and collected for RNA and protein extractions.

3.5.10 RT-PCR

RNA was isolated from cell lines using Trizol reagent (Invitrogen) as per the manufacturer's protocol. An aliquot of 5 µg RNA was then used for cDNA synthesis using the SuperScript first strand cDNA synthesis kit (Invitrogen). RT-PCRs were carried out using using ABI Prism 7900HT sequence detection systems with Applied Biosystems TaqMan Gene expression assays (p21 (CDKN1A) :Hs01121172_m1; MYC:Hs99999003_m1; TP53:Hs00153349_m1; MDM2: Hs01066938_m1)

3.5.11 Northern Blotting

Total RNA was extracted with TRIZol solution (Invitrogen) and the integrity of RNA was assessed with an Agilent BioAnalizer 2100 (Agilent, Palo Alto, CA, USA). Northern blotting was performed as described by Calin et al., 2002. The oligonucleotides used as probes were the complementary sequences of the mature miRNA (miRNA registry).

3.5.12 Cell viability assay and Apoptosis assay

Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO2 incubator. Cell viability was examined with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-Cell Titer 96AQueous One Solution Cell
Proliferation Assay (Promega), according to the manufacturer’s protocol. Metabolically active cells were detected by adding 20 µl of MTT to each well. After 1 hr incubation, the plates were analyzed in a Multi label Counter (Bio-Rad Laboratories). Apoptosis was assessed using Annexin V–FITC apoptosis detection kits followed by flow cytometric analysis. For Annexin V staining, MM cells (pre-miRNAs pool or Scrambled transfected) were treated with MI-219 at different concentrations (0, 2.5, 5.0, 10 µM) for 24 hr and then treated as for DNA content analysis, except that fixation was omitted and the Cells (5×10^5 per sample) were resuspended in PBS containing 25 µg/ml Annexin-V-FLUOS (Roche Applied Science) and 50 µg/ml PI prior to FACS analysis. The percentage of apoptosis indicated was corrected for background levels found in the corresponding untreated controls. The percentage of apoptotic cells was expressed as the mean± SD of three experiments.

3.5.13 Colony assay

A total of 30x10^3 cells were infected with Lenti-mir-192: PMIRH 192-PA-1 Lenti-mir-194-1:PMIRH 1941PA-1; Lenti-mir-215:PMIRH 215 PA-1; Control Lentivector(pCDH-CMV-EF1-copGFP cDNA cloning and Expression vector): CD511 B-1(System Biosciences) as per the manufacturer's protocol. MM cells were plated in quadruplicate in 1mL of methylcellulose medium for Mouse cells (Cat#03234, Stem cell Technologies) in 6-well culture plates. Colonies consisting of more than 40 (125 µ) cells were scored at 14 days.

3.5.14 Cell cycle analysis
Nocodazole was (Sigma-Aldrich) was dissolved in DMSO as a stock solution of 10 mg/ml for cell cycle arrest in G2/M phase. Cells were first arrested and synchronized in G2/M phase by growth in 80 nM nocodazole for 16 hr. Cells were then washed and fresh medium added. After 6 hr, cell cycle analysis was performed by propidium iodide staining. Corresponding amounts of DMSO alone were added in control experiments. In experiments involving transfection and MI-219 treatment, the cells were first transfected, incubated for 24 hr, and then treated with the chemotherapeutic drug for 24 hr. For DNA content analysis, cells were fixed in methanol at -20° C, washed again, rehydrated, re-suspended in PBS containing 50g/ml propidium iodide (PI) and 50 µg/ml RNase A, and analyzed by flow cytometry (Becton Dickinson). For detection of caspase 3 activity, KMS28BM and MM1s cells were cultured in 96-well plates and treated with Nutlin-3a. After the treatment the cells were analyzed using Caspase-Glo 3 Assay kit (Promega) according to the manufacturer’s instructions. Continuous variables were expressed as mean values± standard deviation (s.d.).

3.5.15 Transendothelial migration assay

IGF-I–induced MM transendothelial migration was determined using 24 well, 6.5 mm internal diameter transwell cluster plates with polycarbonate membranes (5 µM pore size) separating the 2 chambers (Corning Costar, Cambridge, MA). Bone marrow stromal cell lines HS-5 and HS-27A were grown on the insert for 24 hrs to produce a confluent monolayer. IGF-I or SDI-1á diluted to varying concentrations in RPMI 1640 was loaded in the lower chamber. MM cell suspensions starved for 3 hrs in serum-free RPMI 1640 were loaded onto the insert (upper chamber). Plates were then incubated for 4 hr at 37° C.
At the end of the incubation period, cells migrating through endothelial or bone marrow stromal cell layers into the lower chamber were harvested, stained with trypan blue, and counted under a microscope.

3.5.16 Chromatin Immunoprecipitation assay

Chromatin immunoprecipitation was performed as described by de Belle et al., 2000 with slight modifications. Cells (5×10^6) from MM1s treated with Nutlin-3a were fixed in 1% formaldehyde for 10 min at 37°C for chromatin cross-link. Cells were washed with ice-cold 1× PBS, scraped in 1×PBS plus protease inhibitors, and collected by centrifugation. Cell pellets, resuspended in cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 1% SDS] plus protease inhibitors. The probes were sonicated 25x for 30 s with a Bioruptor sonicator (Diagenode) and pelleted. The supernatant was diluted with dilution buffer [17 mmol/L Tris (pH 8.0), 167 mmol/L NaCl, 1.2 mmol/L EDTA, 1.1% (v/v) Triton X-100, 0.01% (w/v) SDS].DNA-protein complexes were immunoprecipitated using 5 µg of the anti-p53 antibody (Santa Cruz) or with mouse polyclonal IgG control (Zymed). Cross-links in the immunoprecipitated chromatin were reversed by heating with proteinase K at 65°C overnight, and DNA was purified by the MinElute Reaction Cleanup column (Qiagen) and resuspended in water. The purified chromatin was subjected to PCR and the products were analyzed by gel electrophoresis using 2% agarose. The following primers were used: p53 binding site in miR-194-2-192 cluster promoter For: 5’-TGGGTGGGTCCATGGGAAC-3’; Rev 5’-GCTTTCTGCTCTGTC CCAGT-3’. Negative control for miR-194-2-192 cluster promoter region For: 5’-AGGCCCTGGAGGAGACC AG-3’; Rev: 5’-

3.5.17 Immunofluorescence

The effect of miRNAs pool (miR-192, miR-215) or scrambled sequence on MM samples (n=9) was assessed by immunocytochemical method. At 24 hr after transfection cells were attached to the slide by cytospin technique. Briefly, cells were fixed and permeabilized by incubation in ice-cold acetone and the washed in PBS. Cells were incubated for 1 hr with 5% BSA and then incubated over-night with 1:100 diluution in PBS of IGF-1R and IGF-1 antiserum (Santa Cruz Biotechnology; Cell Signaling) and then incubated with Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes). The slides were mounted in mounting medium for fluorescence with DAPI (Vector, Burlingane, CA) and visualized using an epifluorescence microscope (Nikon Eclipse E800; Nikon, Avon, MA) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX), as previously described.

3.5.18 Statistical Analysis
Student's t test and one-way analysis of variance was used to determine significance. All error bars represent the standard error of the mean. Statistical significance for all the tests, assessed by calculating p value, was <0.05. Spearman correlation coefficient was calculated to test the association between miR-192 and MDM2 mRNA in MM samples (n=33). Expression values (obtained by qRT-PCR) from the 4 healthy PCs, 14 MGUS and 33 MM samples for each of the 3 miRNAs (miR-192, miR-215 and miR-194) were tested using the Bartlett test to evaluate the homogeneity of the variance among the samples. Kruskal-Wallis was used to assess whether the 3 miRNAs are differentially expressed among normal PCs, MGUS and MM samples on the basis of the Bartlett test P value. The Kruskal-Wallis test was used for Bartlett test P values less than .001.

3.5.19 Detection of tumor progression by bioluminescence imaging

Mice were injected with 75 mg/kg of Luciferin (Xenogen, Hopkington, MA), and tumor growth was detected by bioluminescence 10 min after the injection. The home-built bioluminescence system used an electron multiplying CCD (Andor Technology Limited, Belfast, United Kingdom) with an exposure time of 30 sec, and an electron multiplication gain of 500 voltage gain x 200, 5- by-5 binning, and with background subtraction. Images were analyzed using Image-J software (National Institutes of Health, Bethesda, MD).

3.5.20 In Vivo Experiments

For the sub-cutaneous engraftment model 8 wk old male athymic nu/nu mice (Charles River Laboratories, Wilmington, MA) were maintained in accordance with IACUC procedures and guidelines. 8 x 10^6 of GFP/Luc + MM1s cells were suspended in 0.10 ml
of extracellular matrix gel (BD Biosciences) and the mixture was injected subcutaneously into the right flank. 3 wks after injection, mice with comparable size tumors, as detected by bioluminescence images, were treated for 2 wks with a combination of oral dose of MI-219 (200 mg/kg) or vehicle, once a day for 14 days and miRNAs or scrambled sequence oligos (10µg) (Ambion), injected directly into the tumors once a week for 2 wks. Measurements of xenograft growth were performed, and tumor volume was estimated using the formula $4/3 \times (L \times W \times H)/8$. Tumor size was assessed by digital caliper. For the NOD-SCID engraftment model Luc+/GFP+ MM.1S cells (pre-miR-192, 194, 215 or Scr-transfected, as described above) ($8 \times 10^6$ /mouse) were injected into the tail vein of SCID mice. Treatment started 7 days from tumor cell inoculation, by weekly i.v. injections of miRNAs or scrambled sequence. RNA oligos (Ambion) (10 µg) for four cycles (4 wks). Tumor size was assessed every 7 days by bioluminescence images. Thirty-five days after injection, mice were analyzed by bioluminescence images and then sacrificed. MM1s bone marrow isolated cells were stained with anti-human CD-138 antibody (BD) and analyzed by FACS analysis. Statistical significance of differences between control and treated animals was evaluated using Student's t test. Animal experiments were conducted after approval of the Institutional animal care and use committee, Ohio State University.

3.5.21 Combined Bisulfite Restriction Analysis (COBRA)

COBRA analysis was performed largely as described (Xiong and Laird, 1997). A sample of 1 µg of genomic DNA was modified with sodium bisulfite using the CpGenome modification kit (Intergen, Oxford, UK) as per the manufacturer's instructions. PCR
products were digested with a restriction enzyme specific for the methylated sequence after sodium bisulfite modification. For the CpG island primers, digestion of the total PCR products was carried out with 20 U BsiEI (New England Biolabs, Hitchin, UK) in 1x manufacturer's buffer supplemented with 100 µg/ml bovine serum albumin for 2 hr at 60°C. For the promoter primers, digestion of the total PCR products was carried out with 20 U TaqI (Invitrogen, Paisley, UK) for the region R1. Digested PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining on GelDoc 1000 (Bio-Rad, Hemel Hempstead, UK). The primers used for the PCRs (and positions relative to the transcriptional start site) were: for the CpG island, miR-192 Region1 For: 5'-GGGTATTGGGAATAGAGAA-3'; Reverse: 5'-CACCCTTCAAAAAATACCTA-3'.

3.5.22 Luciferase Reporter Vector

HDM-2, IGF-1R, IGF-1 3'UTR containing predicted microRNA binding site were amplified by PCR from genomic DNA (293T/17cells) using AccuPrime Taq DNA (Cat no. 12346-086, Invitrogen, Carlsbad, CA) and inserted into pGL3 control vector (Promega) by using Xba1 site immediately downstream from the stop codon of firefly luciferase. Deletion of the first six nucleotides of each complementary seed-region complementary site were inserted in mutant construct using quick change site directed mutagenesis kit from Stratagene (Cat#200517-5, Cedar Creek, TX), according to the manufacture’s protocol. The primers sequences are listed in the supplementary information (SI). In case of promoter assay, miR-194-2-192 cluster promoter were amplified by PCR from genomic DNA (293T/17cells) (primers are listed in the
supplementary information) and cloned into pGL3 basic vector (Invitrogen) by using SacI- XhoI sites. To obtain miR-192-2-192 cluster promoter constructs with point mutations in p53 binding site directed mutagenesis kit from Stratagene (Cedar Creek, TX) was used (primers listed below).

3.5.23. List of primers used in this experiment

MDM2 3’UTR primers:

MRE (2117-38) for miR-194

For: 5’-ATTCTAGAAATTCTTGGCTGGACATGGT-3’
Rev: 5’-ATTCTAGATCAAGTGAGAAATGCCTCAA-3’

MRE (3495-4497) for miR-192/215:

For: 5’-ATTCTAGATCCAGCTTAGTTTCAGA-3’
Rev: 5’-ATTCTAGAGATGCGATCAAACATCC-3’

MRE (5974-95) for miR-194:

For: 5’-ATTCTAGACAATAATGGCCCAAAGGGATT-3’
Rev: 5’-ATTCTAGACTTCAAGCTGCCCAGTGATA-3’

MRE (6360-80) for miR-192/215

For: 5’- ATT TCTAGACAATAATGGCCCAAAGGGATT-3’
Rev: 5’-ATT TCTAGACAAAAGCTAGTCCCCGTCTG-3’

Full (2117-6380):

For: 5’-ATTICTAGAAATTCTTGGCTGGACATGGT-3’
Rev: 5’-ATT TCTAGACAAAAGCTAGTCCCCGTCTG-3’

Deletion primers for MDM2 3’-UTR upon request.
MRE for miR-192/215

F: 5’-ATTTCATAGAGAAGCTGAAAGATGCACTG-3’
R: 5’-ATTTCATAGAGCAGCCACAGACATGAGAT-3’

**IGF1R 3’UTR primers:**

MRE (4600-5514) for miR-192/215
For: 5’-ATTTCATAGAATCCATTCACAAGCCTCCTG-3’
Rev: 5’-ATTTCATAGA CCTTCCCATCTGTGTTCCTG-3’

MRE (6013-7572) for miR-192/215:
F (4600-5514): 5’- ATTTCTAGATTTTGCTGGTCAGCAGTTTG -3’
R (6913-7572): 5’- ATTTCTAGATCCATCTGCACAGAAGCAGT-3’

Deletion mutagenesis

**IGF1 Deletion**

For: 5’-TTAATTGACCATACTGGATACTATTATTCTGTTCCTCTCTTCCCAA-3’
Rev: 5’-TTGGGGAAGAGAGAACAGAAATAGTATCCAGTATGGTCAATTAA-3

**IGF1R Deletion**

For(4600-5514): 5’TGTACACACCCGCCTGACACCATTACAAAAAACACGTGG3’
Rev(4600-5514): 5’CCACGTTTTTTTTGTGAATGTTGTTCAGGCAGCTGGTGTAAC-3’
For(6913-7572): 5’TTTCTCTGTTCTAGGACTTTACAGTTCTATGTGTAGACC3’
Rev(6913-7572): 5’GGTCTAACATAGAACTGTAAGAGTCCTAGGAACAGA-3’

**miR-194-2-192 promoter primers:**

1. (-1871- +186)
F: ATTGAGCTCCCTACGACACAGTGCGAGAGG
R: ACTCTCGAGGGAAACCAAGGCGACAGAGGAA

2. (-1104)
F: ATTGAGCTCCAGCCCCCTCTCAGATCCTC

3. (-958)
F: 5’ATTGAGCTCATCAGGGCACAGGGGAGACCA3’

4. (-912)
F: 5’ATTGAGCTCCTCTGTGGGCTCTGCCTTGCCC3’

5. (-631)
F: 5’ATTGAGCTCCAGCTCCAGCACTTGGAGGG3’

6. (-530)
F: 5’ATTGAGCTCATGCCCCCCACACATCTTG3’

7. (-481)
F: 5’ATTGAGCTCCCCTGCCCTGCTTCAGTG3’

8. (-429)
F: 5’ATTGAGCTCGAAGCAGGCTCGGGTTGGG3’

9. (-339)
F: 5’ATTGAGCTCGGACAGCTGGGGCAGCAGGCT3’

10. (-245)
F: 5’ATTGAGCTCGGACAGCTGGGGCAGCAGGCT3’

11. (-125)
5’ATTGAGCTCTCTTGACCCGCCCCACCTGC3’
Mutation primers for p53 binding sites in miR-194-2-192 cluster promoter

1. 192-Exch1 (F)
5’-CCAGCCTGATGCTTCCCTGGATCCTCCCCACCCCTGCCCCGGCA-3’

2. 192-Exch1 (R)
5’-TGTCCTGGCCGGGAGGATCCAGGAAGCATCAGGCTGG-3’

3. 192-Exch2 (F)
5’-GCCTCCTGGACCAGCCCACTTCCCTCCGGCACATGAGCAGGCT-3’

4. 192-Exch2(R)
5’-AGCCCGTGGACCTTGCCCGAAGAGTGCCGGGCTCCAGGAAGC-3’

5. 192-Exch3 (F)
5’-GCCTCCTGGATCCTCCCCCACTTCCCTCCGGCACATGAGGCT-3’

6. 192-Exch3 (R)
5’-AGCCCGTGGACCTTGCCCGAAGAGTGGGGAGGATCCAGGCT-3’

We have two putative sites for binding. Primer 1 and 2 were used for first two point mutations in the first putative p53 binding site (listed 1 and 2 in Figure 2B), primer 3 and 4 were used for the second two points mutation (listed 3 and 4 in Figure 2B) in the second p53 putative binding site and primer 5 and 6 were used for all mutations of first and second putative sites at the same time.
Figure 3.1 Identification of p53-Regulated miRNAs in MM Cells

(A) Overview of two-way (genes against samples) hierarchical cluster (Euclidean distance) of 6 MM cell lines in duplicate using the genes that vary the most between samples. As shown, the clustering is mainly determined by the presence of WT TP53 expression (NCI-H929, MM1s, and KMS28BM) or mutant/null TP53 (U266, RPMI-8226, JJN3) in the cell lines. (B) Overview of two-way of MM1s cells treated with 10 mM Nutlin-3a overnight (biological quadruplicate) and with DMSO (biological triplicate) using the genes that vary the most between samples. As shown, the clustering is mainly determined from the Nutlin-3a treatments and DMSO treatment. (D). The PCR products were normalized to ACTIN expression. Values represent mean observed in four different studies ±SD. (E) Kinetics of miR-194, miR-192, miR-215, and miR-34a in MM1s cells after Nutlin-3a treatment, measured by qRT-PCR and northern blot analysis. Lines represent relative fold changes between DMSO and Nutlin-3a treatment ±SD. RNU44 (qRT-PCR) and RNU6B (northern blot) expression was used for normalization. (F–H) miR-192, miR-215 and miR-194 relative expression in CD138+ PCs from healthy, MGUS, and MM samples (see Table 3.3) were determined by Taqman q-RT PCR assay. Each data sample was normalized to the endogenous reference RNU44 and RNU48 by use of the 2– ct method. The relative expression values were used to design box and whisker plots. Dots in the boxes indicate outlier points. Kruskal-Wallis analysis assessed that the three miRNAs were differentially expressed among MGUS samples versus MM PCs samples of the Bartlett test p < 0.001.
Figure 3.2 miR-34a, miR-194 and miR-192 expression are related to TP53 status in MM cells. (A-B) p53 and MDM2 expression in MM cell lines used for microarray experiments. (C) miR-34a, miR-194 and miR-192 relative expression in WT TP53 and Mutant/Null TP53 cells. (D) Kinetics of activation of miR-15a, 29a and 29b in MM1s cells upon Nutlin-3a treatments, measured by qRT-PCR and Northern blot analysis. (E) Time course of MYC mRNA expression in Nutilin-3a treated MM1s cells by RT-PCR. (F-K) miR-192, miR-194 and miR-215 re-expression is dependent on p53 activation. (F), RPMI-8226 (Mut TP53) (G) and U266 (Mut TP53) (H) cell lines after different times of Nutlin-3a treatment. (I) RPMI-8226 (J) and U266 (K) cells Nutlin-3a treated compared to DMSO treatment. The PCR products were normalized to RNU6B expression. L) Representative fax analysis of purified CD-138+ plasma cells. M) Western analysis showing p53 and MDM2 expression after Nutlin-3a overnight treatment. (N) The PCR product was normalized to ACTIN mRNA expression. (O) miR-194, miR-192, miR-215 and miR-34a expression in primary tumor samples, after Nutlin-3a treatment, measured by stem loop qRT-PCR. The bar graph in Figure N and O are representative of 8 samples used for primary culture and Nutlin-3a treatments.
Figure 3.3 miR-194-2-192 Cluster Is Induced Following p53 Activation

(A) Luciferase reporter activity of promoter constructs of miR-192-194-2 cluster on chromosome 11q13.1 in MM1s cells after p53 transfection ±SD. (B) Relative luciferase activity of P7 reporter construct. Deletions introduced into the P7 construct are shown in yellow (X) showing abolition of the promoter activity. (C) Chip assay showing binding of p53 to the miR-192-194-2 cluster promoter in vivo in MM1s cells. (D) Luciferase activity of empty vector (EV), P2 and P10 reporter constructs after nongenotoxic activation of p53 and MDM-2 mRNA silencing. Values represent mean ± SD from three experiments.
Chip assay with anti-p53 or normal IgG from the same animal after 24 hr of p53 non-genotoxic activation, revealed binding of p53 to the miR-194-1-215 cluster promoter in vivo in MM1s cells. ChIP primers were designed to amplify the region containing the putative p53 binding site in the pri-miR-194-1-215 promoter (~2.7 kb from the cluster). p53-responsive CDKN1A gene promoter associated with p53 was used as positive control, whereas amplification of a MT-RNR2 gene portion yielded very little background signals and served as negative control.
Figure 3.5 miR-192, 194 and 215 regulate CDKN1A and MDM2 mRNA levels in MM cells. (A-C) MM1s, NCI-H929, KMS28BM and RPMI-8226 cells (pre-miRNA-192, 194, 215, Scr sequence–transfected) were harvested at 48 hr after transfection and CDKN1A (A), TP53 (B) and MDM2 (C) mRNA expression level was assessed. The PCR products for the genes were normalized to ACTIN mRNA expression. The bar-graphs represent mean values observed in four separate studies ± SD. (D) miRNA-192-194 and 215 effects on MDM2 protein level in Mut TP53 cells (RPMI-8226). RPMI-8226 cells (miRNA-192, 194, 215, Scr sequence-transfected) were harvested at 72 h after transfection. Whole cell lysates were subjected to Western blot using MDM2 and Gapdh antibodies. Bars indicate MDM2 protein relative fold change ±SD. Gapdh was internal loading control and used for the densitometry analysis. The experiment was performed in triplicate.
**Figure 3.6 miR-192-194-215 Induce Decrease of Proliferation and Cell-Cycle Arrest in WT TP53 MM Cells.** (A–D) MTS assay performed in MM1s (A), NCI-929 (B), KMS28BM (C), and RPMI-8226 (D) cell lines. Cells were transfected with miR-192, 194, 215, and scrambled sequence (Scr) and were harvested at 24, 48, and 72 hr after transfection. p values are indicated. (E and F) Soft agar colony suppression assay in WT TP53 and mutant TP53 MM cell lines after miRNAs transduction by lentivectors. (G–J) Flow cytometry analysis in MM1s (G), NCI-H929 (H), and KMS28BM (I) cells (miR-192, 194, 215, and Scr transfected) at 48 hr of transfection, after first being arrested and synchronized in G2/M phase by Nocodazole for 16 hr. Apoptosis in KMS28BM was evaluated by caspase-3 activity (J). All experiments were performed in triplicate ±SD.
Figure 3.7 miR-192, 194, and 215 target human MDM2 (HDM2)

(A-B) Assessment of expression of miRNAs in MM transfected cells (B) were harvested at 72 hr after transfection (C) MDM2 mRNA (HDM2) (D-G) miRNAs predicted to interact with HDM2 mRNA in its 3′-UTR (H-J) and CS3975 and CS6360 constructs for miR-192 and 215 (I-K).
Figure 3.8 miRNA-192-194 and 215 Target MDM2 at the mRNA and Protein Levels in MM Cells. (A) MM1s and NCI-H929 cells (pre-miRNA-192, 194, 215, Scr sequence-transfected) were harvested at 72 hr after transfection and 12 hr Nutlin-3a treatment (10 mM). Whole-cell lysates were subjected to western blotting using p53, MDM2, p21, and Gapdh antibodies. Densitometric analysis showing the effect of miR-194 (white bars), miR-192 (gray bars), miR-215 (black bars) compared with Scr sequence (green bars) transfected cells of endogenous p53, MDM2, and p21 in MM1s ±SD (B) and NCI-H929 (C) Nutlin-3a treated. (D) Immunoblot analysis after 48 hr of miR-192, miR-194, miR-215 (pool), and Scr ASOs transfection in MM1s and NCI-H929 cells after 12 hr of treatment with 10 mM Nutlin-3a; Gapdh was internal loading control and densitometric analysis was reported ±SD (E). (F) MDM2 mRNA expression normalized for GAPDH mRNA expression in MM1s and NCI-H929 cells miRNAs or Scr transfected after Nutlin-3a treatment (6–12 hr) ±SD. (G) miRNAs predicted to interact with HDM2 gene in several consensus binding sites (XXX) at its 3’UTR. All experiments were performed in triplicate ±SD. (H) MDM2 mRNA relative expression in CD138+ PCs from healthy, MGUS, and MM samples with determined by RT-PCR. Each data sample was normalized to the endogenous reference ACTIN by use of the 2–ct method. Kruskal-Wallis analysis assessed that MDM2mRNA is differentially expressed among the healthy and MGUS samples vs MMPCs samples of the Bartlett test P value (<0.01) (see Table 3.3). (I) Graphic of the negative Spearman correlation coefficient (r = -0.698) corresponding to a decreasing monotonic trend between log of MDM2 mRNA relative expression and log of miR-192 relative expression (p < 0.001, N = 33).
Figure 3.9 miR-192, 194, and 215 Increase Sensitivity to MI-219 In Vitro and In Vivo by Targeting MDM2

(A) Effects of miR-192, 194 and 215 on endogenous p53, p21, and MDM2 levels (western blots) in MM1s cells treated with MI-219 at different concentrations. Densitometric analysis for p53 in untreated cells and for p53 and MDM2 protein levels in 2.5, 5, and 10 mM MI-219-treated cells is reported (B). All experiments were performed in triplicate ±SD. (C) Apoptotic effect at different concentrations and time points for each miRNA-transfected cells was assessed by caspase-3 activation assay ±SD. (D) Apoptosis associated with the pool of these miRNAs upon MI-219 treatment (24 hr) at different concentration (2.5–10 mM) was evaluated by Annexin V. All experiments were performed in triplicate ±SD. (E) Gfp/Luc + MM1s cells were injected subcutaneously into the flanks of nude mice; at 3 weeks postinjection, mice with comparable tumor sizes were selected for treatment (untreated). In vivo confocal imaging of GFP+/Luc+MMcells engrafted in athymic nu/nu mice after 2 weeks of combined treatment with oral MI-219 or vehicle (VE) plus pre-microRNA pool or Scr sequence directly into the tumors. Graphic represents the mean of tumors value (mm2) before (3 weeks) and after the treatment (3+2 weeks) ±SD.
Figure 3.10 miR-192 and 215 target IGF-1 and IGF-1R in MM cells

A-D) Effect of Nutlin-3a treatment on IGF-R and IGF-1 protein expression in MM cells with different TP53 status. WT TP53 (MM1s and NCI-H929) (A-B) and Mutant TP53 (RPMI-8226 and U266) (C-D) cells were treated with Nutlin-3a (10 µM) or DMSO vehicle and whole cell lysates collected at different time points were immunoblotted using antisera against IGF-1R, IGF-1, p53, MDM2. Gapdh was used as loading control. A decrease in IGF-R and IGF-1 protein level is shown only in TP53 WT cells upon Nutlin-3a treatment. E) Representation of the full length IGF-1R mRNA. F-G) miRNAs predicted to interact with IGF-1R gene in several consensus binding sites at its 3′-UTR, according to “in silico” RNA-22 prediction software with a folding energy > -27 Kcal/mol. H-I) Luciferase assay showing decreased luciferase activity in cells co-transfected with 2 different constructs (1 kb each) of pGL3-IGF-1R-3′UTR and miR-215 (H-I) and miR-192 (I) but not with miR-194 and Scr sequence (H-I). Deletion of six bases in all putative consensus sequences abrogates this effect (Del) (H-I). Bars indicate firefly luciferase activity normalized to Renilla luciferase activity ± SD.
Figure 3.11 miR-192-215 Regulate IGF-1 and IGF1-R Expression in MM Cells

(A and B) Western blot showing IGF-1R and IGF-1 expression after miR-192 and miR-215 transfection using pre (A) and ASOs (B) for miR-192, 215, 194, and Scr in MM1s cells treated for 12 hr with Nutlin-3a. (C) Western blots after IGF-1 knockdown in MM1s (si-RNA) using anti-IGF-1R, IGF-1, and Gapdh antibodies. (D and E) miRNAs predicted to interact with IGF-1 and IGF-1R gene at their 3’UTR, according to “in silico” Target Scan (IGF-1) and RNA-22 (IGF-1R) prediction software. Luciferase assay showing decreased luciferase activity in MM1s cells cotransfected with pGL3-IGF-1-3’UTR (D) or pGL3-IGF-1R-3’UTR (full) (E) and miR-192, 194, 215, or Scr. Deletion of six bases in all putative consensus sequences on IGF-1-3’UTR abrogates these effect (Del) (D). Bars indicate relative luciferase activity ±SD. All experiments were performed in triplicate. (F and G) Immunofluorescence using anti-IGF-1R (F) and anti-IGF-1 (G) in red and blue nuclear DNA, from CD-138+ PCs from nine MM patients transfected with miR-192 and miR-215 (pool) or Scr and intensity of the signal was assessed ±SD. (H) The efficiency of the transfection in the nine samples was evaluated using fluorescent double-strand RNA oligos. Scale bars indicate 25 mM.
Figure 3.12 miR-194, 215, and 194 Block Invasion Ability of MM Cells

(A) MM1s and RPMI-8226 cells were harvested 72 hr after transfection. (B) Intraepithelial migration assay in MM cells miRNAs transfected using HS-5 cells at different concentrations of IGF-1 as attractant. Bars indicate relative fold change of migration compared with the control ±SD. (C and D) In vivo confocal imaging. (E) Bone marrow cells from the mice used for the experiment were isolated and human CD-138 positive cells (engrafted cells) were detected using anti-CD-138 antibody by flow cytometry (P2 fraction).
Figure 3.13 miR-192 and 215 affect the ability of MM cells to adhere and migrate in response to IGF-1

(A-B) MM1s and RPMI-8226 cells after transfection were harvested, treated with calcein and incubated with IGF-1 (50 ng/ml). (C) Intra-epithelial migration assay in MM1s and RPMI-8226 cells using HS-27A stromal cell as cellular layer at different concentrations of IGF-1 as attractant. Bars indicate relative fold change of migration compared with the control. All experiments were performed in triplicate ± SD.
Figure 3.14 The promoter region of miR-194-2&192 is methylated in MM cell lines

(A) Representation of the genomic region of miR-194-2&192 obtained from University of California Santa Cruz genome browser (2006). The red arrow is the region that we analyzed for the methylation study, including the p53 consensus sequence. (B) Combined bisulfite restriction analysis (COBRA) in 9 MM cell lines. Universal methylated DNA from Millipore was used as positive control and normal CD-138+ plasma cells as negative control. The digestion of PCR products coming from methylated DNA was carried out with TaqI for the region R. (C) stem-loop q-RT-PCR for miR-192 and miR-194 and RT-PCR for SOCS-1 genes—normalized to RN44 and ACTIN respectively, expressed as fold increases after 3 days of treatment with 5-Azagctidine (10 µM) compared to DMSO treated cells. All experiments were performed in triplicate. ± SD. (D) Illustration of the p53—miR-192,194,215—MDM2 auto regulatory loops, showing the central role played by the miRs in determining the balance of p53 suppressor and the MDM2 oncoprotein expression levels.
Figure 3.15 miR-192, 215, and 194 Impair the p53/MDM2 Autoregulatory Loop

Model to illustrate the possible role of miR-192, 194, and 215 in control of MDM2 and IGF-1/IGF-1R pathways in MM cells.
Table 3.1

miRNAs differentially expressed between WT TP53 vs Mutant TP53 MM cell lines

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Table 3.2
miRNAs differentially expressed in Nutlin-3a treated vs MM1s cells DMSO-treated
MM1s cells.

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CHAPTER 4

MICRORNAS/TP53 FEEDBACK CIRCUITRY IN GLIOBLASTOMA MUTIFORME
4.1 ABSTRACT

MicroRNAs (miRNAs) are increasingly implicated in regulating cancer initiation and progression. In this study two miRNAs, miR-25 and -32, are identified as p53-repressed miRNAs via p53-dependent, negative regulation of their transcriptional regulators, E2F1 and MYC. On the other hand, miR-25 and -32, which are upregulated in glioblastoma multiforme (GBM), result in p53 accumulation by directly targeting MDM2 and TSC1, negative regulators of p53 and the mTOR pathway, respectively, leading to inhibition of cellular proliferation through cell cycle arrest. Thus, there is a recurrent autoregulatory circuit involving expression of p53, E2F1 and MYC to regulate the expression of miR-25 and -32, microRNAs that in turn control p53 accumulation. Significantly, overexpression of transfected miR-25 and-32 in GBM cells inhibited growth of the GBM cells in mouse brain in vivo. The results define miR-25 and -32 as positive regulators of p53 underscoring their role in tumorigenesis in glioblastoma.
4.2 INTRODUCTION

Glioblastoma multiforme (GBM) is by far the most common and aggressive tumor of the central nervous system. Despite recent improvements in surgery, radiation therapy and cytotoxic chemotherapy, the prognosis for GBM remains grim, with median survival time less than 1 year after diagnosis. Of all glial tumors, GBM seems to exhibit the greatest number of genetic changes (Nigro et al, 1989). The TP53 tumor suppressor gene, a transcription factor for numerous genes involved in cell cycle control, DNA repair, apoptosis, and angiogenesis (Shangary et al, 2008; Shats et al, 2004), is one of the most frequently mutated gene in human cancer. Given its profound effects in either inhibiting cell proliferation or inducing apoptosis, the expression levels of the TP53 gene product, p53, is tightly controlled through a feedback loop involving the p53 downstream target gene, MDM2, that negatively regulates p53 through Mdm2-mediated ubiquitination of p53 (Xirodimas et al, 2001). As such, even modest changes in Mdm2 level can perturb p53 protein level and affect the tumorigenesis process. In addition, overexpression of MDM2 is a common observation in glioblastoma multiforme (He, 1994; Korkolopoulou et al, 1997).

miRNAs, small non-coding RNAs, of about ~22 nucleotides that mediate post-transcriptional silencing of specific target mRNAs, are being increasingly recognized as an important determinant of tumor development and progression (Calin and Croce, 2006).
Deregulated miRNAs were suggested to exert their function in cancer through silencing of key cell-fate regulators by directly binding their 3’UTR (Johnson et al, 2005; Petrocca et al, 2008). Furthermore, miRNAs cooperatively function with certain transcription factors (TFs) in the regulation of mutual sets of target genes, allowing the coordinated modulation of gene expression both transcriptionally and post-transcriptionally. Specially, it has been revealed that there is a recurring network motif in which a TF regulates the miRNA with which it cooperates in regulating a common set of targets (Shalgi et al, 2007).

Several studies have implicated p53 in the regulation of miRNAs expression (Chang et al, 2007; Pichiorri et al, 2010; Ravar-Shapira et al, 2007). However, most miRNAs studied so far are positively correlated with p53 expression while miRNAs repressed by this tumor suppressor have rarely been studied. Here, we report the identification of a set of miRNAs repressed by p53, through transcriptional repression of two TFs, E2F1 and MYC, and demonstrate the anti-oncogenic potential of these miRNAs, that induce cell cycle arrest and inhibition of cellular proliferation and tumor growth in vivo. Finally, we delineate a network architecture that includes two transcriptional factors, E2F1 and MYC, and miR-25/32, which directly or indirectly co-regulate mutual genes through p53-dependent manner in GBM.

4.3 RESULTS

4.3.1 Identification of p53-regulated miRNAs in human GBM
To identify p53-regulated miRNAs in GBM cells, we performed NanoString nCounter analysis of microRNA expression, a direct global profiling of individual miRNAs in a single reaction without amplification, in U87 cells treated with or without Nutlin-3a which inhibits the formation of the MDM2/p53 complex and results in activation of p53 (Dicken et al, 2009) (Figure 4.1A; Table 4.1). Unexpectedly, the predominant consequence of p53 induction in this model system was widespread repression of miRNAs expression (17, p53-repressed miRNAs, out of total p53-responsive miRNAs, 31; p< 0.05) (Figure 4.1A; Table 4.1). Among the p53-repressed miRNAs, miR-25, the most strongly downregulated in response to p53 activation (median fold change: -2.23), stood out as an attractive candidate for a role in p53-related functions. Moreover, miR-25 was also identified as a miRNA downregulated by p53 in breast cancer (Brosh et al, 2008). Interestingly, one of the p53-downregulated miRNAs (median fold change; -1.5), miR-32, has the same seed sequence as miR-25, a conserved heptameric sequence, indicating that they are able to target the same transcripts. These observations led us to pursue miR-25 and -32 as interesting targets for further studies. To first validate the profile data, we performed stem-loop qRT-PCR analysis in cells with activated p53; miR-25 and -32 were significantly downregulated upon treatment with Nutlin-3a in p53-activated U87 cells (Figure 4.2A and 4.2C). Both mRNA and protein levels of p21, a downstream gene of p53, were also upregulated (Figure 4.2A and 4.2B). Additionally, expression of two other miRNAs of the cluster with miR-25, miR-106b and miR-93, displayed similar downregulation (Figure 4.2D), confirming that expression of the entire miR-106/93/25 cluster is attenuated (Petrocca et al, 2008). In particular, we found no effect of Nutlin-3a treatment of the p53-silenced cells on expression of miR-25
and -32, while the downregulation in the control cells (Figure 4.1B), provided direct
evidence that p53 was involved in miR-25 and -32 repression.

As further evidence of the functional connection between p53 and miR-25 and -32, we
performed a correlation analysis of p53 and miR-25/-32 level using
immunohistochemistry and in situ hybridization (ISH), respectively, in glioblastoma
tissue microarrays (TMAs) consisting of 70 brain tumor samples (Figure 4.1C-D).
Consistently, it was observed that miR-25 and -32 were rarely expressed in tumors in
which p53 was highly expressed, whereas the tumors with low signal of p53 showed high
expression of miR-25 and -32, confirming the inverse correlation (Figure 4.1C-D). In
addition, we found a strong positive correlation between the expression of p53 and p21 in
co-expression assay (Figure 4.3A-B). These data further support the finding that miR-25
and -32 are p53-repressed miRNAs in vivo.

4.3.2 miR-25 and -32 are overexpressed in human GBM

Our results thus far indicate that miR-25 and -32 were strongly downregulated by
p53 and it is known that miR-25 and- 32 are upregulated in a variety of human cancers in
which TP53 gene is frequently mutated (Ambs et al, 2008; Philippidou et al, 2010;
Pichiorri et al, 2008; Scalpoli et al, 2010). We next investigated miR-25 and -32
expression by ISH in TMAs consisting of 60 brain tumor tissues. The levels of miR-25
and -32 were significantly upregulated in glioblastoma tissues when compared with
normal tissues; the signal of miR-25 and miR-32 (blue) was highly detectable in 85%
(51/60) and 80% (48/60) samples, respectively, compared with normal samples (Figure
4.4A-B). We next examined miR-25 and -32 expression levels in 57 brain tumor
specimens, 7 glioblastoma cell lines and 4 normal human brain tissue samples by qRT-PCR. Using Kruskal-Wallis analysis, we found that miR-25 and -32 were significantly upregulated in brain tumors (Figure 4.4C). Additionally, miR-25 and -32 were highly expressed in tumor tissues as compared with adjacent normal tissues in five patients samples (Figure 4.4D).

4.3.3 E2F1 and MYC transcriptionally activates miR-25 and -32

Next, we were interested in the mechanism of p53 repression of miR-25 and -32 expression. It has been reported that E2F1 regulates the expression of the miR-106b-25 cluster, located in intron13 of the MCM7 host gene (Broth et al, 2008; Petrocca et al, 2008). MYC-dependent regulation of the miR-106b-25 cluster has also been observed (Petrocca et al, 2008; unpublished data by F. Petrocca). In fact, E2F1 can activate MYC transcription and vice versa and they cooperatively modulate the expression of miRNAs (Leone et al, 1997; Matsumura et al, 2003; O’Donnell et al, 2005; Woods et al, 2006). Therefore, we decided to test whether E2F1 and MYC could also be responsible for the transcriptional activation of miR-32, located in the intron14 of the host gene C9orf5. We first investigated the correlation between miR-25/32 and E2F1/MYC expression in p53-induced cells. Consistent with previous data showing that p53 transcriptionally represses MYC expression through the binding to its promoter (Ho et al, 2005), MYC mRNA and protein expression were significantly downregulated in p53-induced U87 cells (Figure 4.5A-B). E2F1 mRNA and protein expression was also strongly reduced (Figure 4.5A-B), along with significant reduction of miR-25 and -32 (Figure 4.2). These data suggest that E2F1 and MYC inhibition by p53 is necessary for the downregulation of miR-25 and -32.
To determine if E2F1 and MYC expression are essential for regulation of miR-25 and -32, we specifically silenced E2F1 or MYC, by RNA interference. Consistently with previous reports that they transactivate each other (Leone et al, 1997; Matsumura et al, 2003), their transcriptional levels were decreased or increased in response to the knockdown or overexpression of each other, respectively (Figure 4.6A and 4.6C). Subsequent qRT-PCR showed that the knockdown of E2F1 or MYC resulted in a reduction of miR-25 and -32 levels (Figure 4.5C). Conversely, overexpression of the individual TFs induced miR-25 and -32 (Figure 4.5D). The levels of miR-93 and -106b expression were also decreased or increased in response to knockdown or overexpression of E2F1 and MYC, respectively (Figure 4.6B and 4.6D).

As mentioned above, miR-25 and -32 are intragenic, located in introns of MCM7 and C9orf5, respectively. In p53-activated U87 cells, MCM7 mRNA was markedly decreased after p53 induction, as was miR-25 expression (Figure 4.2), whereas C9orf5 expression did not change although miR-32 was decreased (Figure 4.6E). We also observed that MCM7 levels were reduced in E2F1 or MYC- silenced cells, but not C9orf5 (Figure 4.6F). These data suggest that MCM7 and miR-25 are cotranscribed, as previously reported (Petrocca et al, 2008), while C9orf5 and miR-32 are not; miR-32 expression is expressed independently of its host gene and it might have its own promoter. Furthermore, we observed that there was not any luciferase activity with plasmid containing ~3kb upstream of the C9orf5 locus (data not shown). Thus, we cloned ~2.6 kb upstream from the 5’ terminus of miR-32 hairpin structure into the pGL3 reporter (Figure 4.5E) for a luciferase assay. We found that a MYC-responsive element, a non-canonical
E-box (CANNTG), is indeed associated with MYC transcriptional activation of miR-32 using a construct mutated for this sequence (Figure 4.5E). Nonetheless, other MYC-responsive domains might also be present in the 2.6 kb fragment since ~30% of luciferase activity was detected with the mutant as compared with full length construct (Figure 4.5E). Notably, we did not observe any luciferase activity in response to E2F1 (data not shown), suggesting that E2F1 might indirectly regulate miR-32 expression via E2F1-transactivated MYC expression. Since we noticed that the miR-32 promoter region was responsive to MYC, MYC-ChIP specificity was confirmed by using MYC siRNA, which resulted in reduced MYC occupation on the miR-32 promoter (Figure 4.5F). In addition, knockdown of MYC led to a reduction of ~50% in luciferase activity for the luciferase reporter construct containing the E-box region (P3) (Figure 4.5G). Collectively, these data confirm that E2F1 and MYC function as transcriptional activators of miR-25 and -32.

4.3.4 miR-25 and -32 stabilize p53 protein to induce cell cycle arrest and inhibit cell proliferation.

It has been reported that miR-25 targets p53 by directly binding to its 3’UTR to result in the reduction of both protein and mRNA levels (Kumar et al, 2011). This led us to test whether p53 could be a direct target of miR-25 and -32 in glioblastoma; we thus introduced miR-25 and -32 into cells with induced p53 expression, to determine if these miRs can suppress the elevated p53. Interestingly and inconsistently with the previous report, p53 accumulated in the presence of miR-25 and -32 when compared with scrambled cells, while p53 mRNA levels did not change (Figure 4.7A; Figure 4.8A). To clarify the basis of p53 protein accumulation in the presence of exogenously expressed
miR-25 and -32, we determined the half-life of p53 protein after treatment with cycloheximide, which blocks protein synthesis, in LNZTA3WT4 (GBM) cells (Figure 4.7B), a p53 tetarcycline-inducible cell line in which p53 is produced in the absence of the antibiotic, but not in its presence (Figure 4.8B). The turnover rate of p53 is normally high in the glioblastoma cells, but in cells expressing miR-25 and -32 p53 became more stable. Of note, p53 mRNA levels did not change upon miR-25 and -32 expression (data not shown). These results indicate that miR-25 and -32 are able to stabilize p53.

To examine the relevance of p53-mediated regulation of miR-25 and -32 in glioblastoma, we tested whether ectopic expression of these miRNAs affected the biology of glioblastoma cells. As shown in Figure 4.7C, rapid growth proliferation was observed only in U251 cells harboring inactivating mutations in p53, while significant growth arrest was shown following transfection with miR-25 and -32 in U87 and LNZTA3WT4 containing wild type TP53. Subsequent experiments indicated that expression of miR-25 and -32 induced a consistent G0/G1 arrest in the two WT TP53 cell lines, U87 and LNZTA3WT4, but not in U251 cells (Figure 4.7D). Together, these data suggest that the function of miR-25 and -32 is likely to be dependent on p53 status in glioblastoma cells. Thus, the results led us to assume that p53 can modulate the function of miR-25 and -32 in glioblastoma. To test this possibility, we performed Western blot analysis to detect p53 protein levels in miR-25 and -32 transfected cells containing functional p53. Consistent with our hypothesis, the levels of p53 protein were increased by miR-25 and -32 compared with scrambled sequence in U87 and LNZTA3WT4 cells (Figure 4.7E). Levels of p21, a p53 downstream transcriptional target, were also increased (Figure 4.7E).
Altogether, these data suggest that miR-25 and -32 expression causes p53 accumulation, which induces cell cycle arrest and inhibits cell proliferation in WT TP53 cells.

4.4.5 miR-25 and -32 target MDM2

To define molecular mechanisms by which miR-25 and -32 cause accumulation of p53, the RNA22 target prediction program was used to discover targets of these miRs; we found that the 3’UTR of the MDM2 gene, a negative regulator of p53, has two predicted miRNA-responsive elements (MREs) containing regions that matched the seed sequences of miR-25 and -32 (Figure 4.9A). To verify that MDM2 is a direct target of miR-25 and -32, MDM2 3’UTR containing MREs was cloned into the pGL3 construct downstream of the luciferase open reading frame. Cotransfection of this construct with pre-miR-25/32 oligonucleotides decreased that luciferase activity as compared to the scrambled oligonucleotides, while the reporter with a mutated seed region did not (Figure 4.10A). In addition, ectopic expression of miR-25 and miR-32, combined with 24hr of Nutlin-3a treatments, led to significantly decreased levels of endogenous MDM2 compared with scrambled cells and increased p53 protein (Figure 4.10B). Because MDM2 protein is rapidly autoubiquitinated and degraded through the proteasome pathway (Marine and Lozano, 2010), p53 induction is necessary for its detection in glioblastoma cells. In contrast, knockdown of miR-25 and -32 by 2’-O-me-anti-miR-25 and -32 increased the protein levels of MDM2 and decreased p53 protein (Figure 4.10C). These findings led us to speculate whether introduction of miR-25 and -32 suppresses p53-dependent MDM2 activation in p53-activated cells. By Western blot analysis, we observed that MDM2 protein levels were significantly reduced in presence of miR-25 and -32 followed by
Nutlin-3a treatment in U87 cells, resulting in accumulation of p53 protein (Figure 4.10D). Furthermore, MDM2 mRNA was strongly reduced in the miR-25 and -32 transfected cells (Figure 4.10E). These results indicate that miR-25 and -32 induce the degradation of MDM2 mRNA, confirming that they regulate both protein and RNA levels. We also found that MDM2 mRNA was significantly upregulated in patient tissues and glioblastoma cell lines compared with normal brain tissues (Figure 4.10F), consistent with previous reports (He et al, 1994; Korkopoulou et at, 1997). Notably, using nonparametric test analysis, we found a significant inverse correlation between miR-25 or -32 and MDM2 mRNA in glioblastoma patient tissues (Figure 4.10G). Together, these data indicate that miR-25 and -32 contribute to p53 accumulation though the direct silencing of MDM2.

4.4.6 miR-25 and -32 directly target TSC1

In a preliminary survey we used several computational algorithms, including TargetScan and PicTar, to search for other target genes of miR-25 and -32; this revealed TSC1 as a predicted target gene of the two miRNAs (Figure 4.11A). Recently, it has been shown that an active mTOR pathway can suppress PI3K-Akt signaling, which affects p53 activity through Akt-mediated phosphorylation of Mdm2 (Chen et al, 2006; Feng et al, 2005; Harrington et al, 2004; Manning, 2004; Manning et al, 2005; Sabatini, 2006). Thus, it is possible that TSC1 suppression by miR-25 and -32 could enhance mTOR activity and induce p53 accumulation in glioblastoma. To verify this possibility, we first examine whether miR-25 and -32 target TSC1 directly by generating luciferase reporters containing its 3’UTR. Luciferase activity dropped 40-50% when this construct were
cotransfected into U87 cells with miR-25 and -32 compared with scrambled oligonucleotides, while the reporter with a mutated seed region did not (Figure 4.11A). In addition, we found that in the presence of miR-25 and -32 TSC1 protein levels decreased in U87 and LNZTA3WT4 cells (Figure 4.11B; Figure 4.9B). On the other hand, inhibition of endogenous miR-25 and -32, using antisense oligonucleotides, led to increased TSC1 levels (Figure 4.11C; Figure 4.9C). In particular, TSC1 protein was increased in knockdown of MYC or E2F1 (Figure 4.11D), which might occur as a result of miR-25 and -32 repression, as confirmed by densitometric analysis in Figure 11E. Next, we wondered if p53-driven down-regulation of miR-25 and -32, would increase the level of TSC1 protein. To this end, we performed Western blot to see TSC1 protein levels in U87 with activated p53. Interestingly, TSC1 was dramatically increased in both U87 and LNZTA3WT4 cells in response to the elevated p53 (Figure 4.11F; Figure 4.9D) without changing mRNA levels (Figure 4.9E). The increased levels of TSC1 were associated with gradual reduction of S6 phosphorylation, a marker of mTOR activation (Figure 4.11F; Figure 4.9D). Conversely, we observed that miR-25 and -32 were sufficient to reduce the elevated TSC1 upon p53 activation when introduced into p53-activated U87 cells (Figure 4.11G), along with the increase of mTOR activity (Figure 4.11G), as shown by densitometric analysis (Figure 4.11H). Taken altogether, these data suggest that TSC1 is a direct target gene of miR-25 and -32 in glioblastoma.

Next, to investigate whether TSC1, as a target gene of miR-25 and -32, is involved in p53 accumulation via the PI3K-Akt pathway in glioblastoma, we first examined the expression levels of genes involved in this mechanism such as S6, Akt, Mdm2 and p53 in miRNAs-transfected cells. As a result of the constitutive activation of downstream signal
transduction of the mTOR pathway, mTOR-mediated phosphorylation of S6 was highly activated in the presence of miR-25 and -32 (Figure 4.11I). In contrast, Akt and Mdm2 activities were markedly reduced, as assessed by phosphorylation (Figure 4.11I), indicating that mTOR activation by miR-25 and -32 may induce the attenuation of Akt and Mdm2 activity, and then to p53 accumulation. To further examine whether TSC1 is essential for miRNAs-mediated p53 activation, we used RNA interference (RNAi) against TSC1 and assayed p53 protein. We observed that the level of p53 protein was increased in response to knockdown of TSC1 and was associated with reduced p-Akt and p-Mdm2 levels (Figure 4.11J-K). Thus, miR-25 and -32 can stabilize p53 through activation of mTOR by targeting TSC1.

4.4.7 miR-25 and -32 suppresses tumorigenicity in vivo

To provide physiological evidence for miR-25 and -32 regulation of tumor suppression in glioblastoma, we compared the antitumor efficacy of miR-25 and -32 in mice bearing intracranial glioma cells, U87 ΔEGFR, a mutant cell line which harbor amplification in the epidermal growth factor receptor (EGFR) gene (Wikstrand et al, 1998). U87 ΔEGFR cells have been widely used in studies of brain tumors because intracranial xenografts of these cells grow much stable and faster than U87 parental cells, U87 (Nishikawa et al, 1994). These cells were transfected with miR-25 and -32 pools, scrambled or treated with PBS as negative control and 2.5x10⁵ U87ΔEGFR cells after the three treatments were implanted into mouse brain and the three mouse groups observed in vivo. The survival of mice in each group (n=6/group) was analyzed by Kaplan-Meier curve (Figure 4.12A). Although control mice treated with PBS died of tumor burden with
a median survival of 18 days, the miRNAs-transfected group showed a significant improvement in survival compared to scrambled group, showing median survival of 28 versus 19 days (Figure 4.12A). In a separate experiment, mice in the three groups were killed 14 days after cell implantation in mouse brain, and the brains were preserved for immunohistochemistry and histopathological analysis. We observed that miRNAs-transfected group had significantly smaller tumor compared to the scrambled or PBS group, indicating that miR-25 and -32 suppressed the tumor growth (Figure 4.12B). Next, to determine if our model of regulation of p53 by miR-25 and -32 was reflected in vivo, mouse brains were stained by immunohistochemistry. We observed that p53 was activated in the miRNAs-transfected group with highly expressed miR-25 and -32 compared with the scrambled group (Figure 4.12C). In addition, TSC1 was significantly repressed in the presence of miR-25 and -32 (Figure 4.12C). The expression levels of Mdm2 mRNA were markedly reduced in the miRNAs-transfected group compared to the scrambled group (Figure 4.12D), consistent with the finding that miR-25 and -32 target Mdm2 mRNA in vitro. In particular, we found co-expression of miR-25 or -32 and p53 in the miRNAs-transfected tumor group (Figure 4.13). Altogether, the data indicate that miR-25 and -32 suppress tumor growth by causing accumulation of p53 protein in vivo.

4.5 DISCUSSION

In the current study, we identified two miRNAs, miR-25 and miR-32, as p53-repressed miRNAs via p53-dependent, negative regulation of their transcriptional regulators, E2F1 and MYC. Our study provides compelling evidence that expression of these miRs causes tumor suppression through mechanisms that lead to accumulation of
p53 protein, resulting in growth arrest in glioblastoma cells. Furthermore, miR-25 and -32 significantly inhibit the tumor growth in vivo. In this process, we revealed that there is a feedback regulatory loop that includes two transcriptional factors, E2F1 and MYC, and miR-25/32, which co-regulate mutual genes in a p53-dependent manner (Figure 4.12E).

Recently, it has been reported that E2F1-inducible miRNAs function as tumor suppressors to suppress cell growth and induce apoptosis (Lize et al., 2009). On the other hand, E2F1 or MYC-dependent miRNAs were involved in cell proliferation and survival by targeting several tumor suppressors (Brosh et al., 2008; He et al., 2005; O'Donnell et al., 2005; Petrocca et al., 2008). In fact, concomitant with promoting cell growth, proliferation, and survival in response to various external stimuli, E2F1 or MYC inhibit terminal differentiation of most cell types, and sensitizes cells to cell cycle arrest and apoptosis in a p53-dependent manner (Evan et al., 1992; Henriksson et al., 2001; Nilsson and Cleveland, 2003; Pelengaris et al., 2002; Sherr and Weber, 2000; Tanaka et al., 2002). This function has attracted in recent years, not least because of its potential role in tumor suppression. Thus, if the p53 pathway is genetically altered and non-functional, the imbalance between proliferation and cell death can lead to tumor development. It is therefore not surprising that this pathway is ablated in many human cancers. Along the same line, we observed that MYC-dependent miR-25 and -32 promote cell proliferation in U251 cells harboring non-functional p53 (Figure 4.7C). Thus, the opposing roles of E2F1 and MYC-dependent miR-25 and -32 in tumorigenesis seems to be dependent upon p53 status; in many human cancers with mutated or strongly downregulated p53, miR-25 and -32 might result in a failure to stimulate enough p53 activity to inhibit tumor
development, and contribute instead to promotion of cellular proliferation and survival.
Furthermore, our observations that miR-25 and -32 target MDM2 and TSC1 in glioblastoma suggest the possibility that those miRNAs render the cells more susceptible to p53-dependent responses. We conclude that miR-25 and -32 are involved in E2F1/MYC-induced p53-dependent stress responses by silencing MDM2 and TSC1.

Given recent advances in the systemic delivery of small RNAs to animal, it would be interesting to determine if the introduction of miR-25 and -32 into tumors can recapitulate the biological effects of p53 protein in many cancers with p53 down-regulation but without inactivating mutations in the TP53 gene itself. Our study provides important insights into the central roles of miRNAs in well-known tumor-suppressor network, the p53 pathway, and may provide a new route to therapeutic miRNA intervention in cancer.

4.6 MATERIALS AND METHOD

4.6.1 In Vivo Experiments

Animal experiments were performed according to the Subcommittee on Research Animal Care of the Ohio State University guidelines and have been approved by the Institutional Review Board. Athymic nude mice, 5-6 weeks old, (Charles River Laboratories, Frederick, MD) were used for all studies. In intracranial tumor study, nude mice were fixed in stereotactic apparatus, and a burr hole was drilled at 2mm lateral to the bregma, to a depth of 3mm. In vivo assay, U87 (∆EGFR) cells were used. Two weeks after tumor cell implantation of U87 (∆EGFR) cells (2x10^5) transfected with scrambled
oligonucleotides, PBS, or mir-25/32 oligonucleotides, three mice from each treatment were euthanized to do immunohistochemistry and in situ hybridization assay and six mice were used in survival test.

4.6.2 MTS assay

Cells were plated in 96-well plates in triplicate and incubated at 37°C in a 5% CO2 incubator. Cell viability was examined with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-Cell Titer 96AQueous One Solution Cell Proliferation Assay (Promega), according to the manufacturer’s protocol. Metabolically active cells were detected by adding 20 µl of MTT to each well. After 1 hr incubation, the plates were analyzed in a Multi label Counter (Bio-Rad Laboratories).

4.6.3 Statistical Analysis

Student's t test and one-way analysis of variance was used to determine significance. All error bars represent the standard error of the mean. Statistical significance for all the tests, assessed by calculating p value, was <0.05. Sperman correlation coefficient was calculated to test the association between miR-25 or 32 and MDM2 mRNA in glioblastoma samples (n=31). Kruskal-Wallis was used to assess whether the two miRNAs are differentially expressed among normal normal brain and glioblastoma samples on the basis of the Bartlett test P value. The Kruskal-Wallis test was used for Bartlett test P values less than .0068 (miR-25) and .001 (miR-32). The in vivo anticancer effect of miRNAs treatments with mice was assessed by plotting survival curves
according to the Kaplan–Meier method, and groups were compared using the log-rank test.

### 4.6.4 Luciferase Reporter Vector

The 3’UTR of the human TSC1 and Mdm2 genes were PCR amplified (primers are listed in the supplementary information). They were then cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega), giving rise to the p3’UTR-TSC1 and p3’UTR-MDM2 plasmids. These constructs were used to generate, by inverse PCR, the p3’UTRmut-TSC1 and –Mdm2 plasmid (primers are listed in the supplementary information). U87 cells were cotransfected with 1 µg of p3’UTR-TSC1 or p3’UTR-Mdm2 and with p3’UTRmut-TSC1 or p3’UTRmut-Mdm2 plasmids and 0.1 µg of a Renilla luciferase expression construct, pRL-TK (Promega), using Lipofetamine 2000 (Invitrogen). Cells were harvested 24 hr after transfection and assayed with Dual Luciferase Assay (Promega) according to the manufacturer’s instructions. Three independent experiments were performed in triplicate.

In case of promoter assay, miR-32 cluster promoter were amplified by PCR from genomic DNA (293T/17cells) (primers are listed in the supplementary information) and cloned into pGL3 basic vector (Invitrogen) by using NheI- XhoI sites.

### 4.6.5 Glioblastoma samples and cell lines

Human U87, U87(∆EGFR), U251 and LNZTA3WT4 cell lines were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum (FBS) and
with 2mM L-glutamine and 100U/ml-1 penicillin-streptomycin. The LNZTA3WT4 cell line was derived from the glioblastoma cell line LN-Z308 that has no endogenous p53 gene expression, due to an internal rearrangement of the endogenous p53 gene. This cell line contains a p53 encoding sequence that is under the control of tetracycline and p53 is produced in the absence of the antibiotic, and is not, in its presence. A total of 4 snap-frozen normal and 57 glioblastoma tissues were collected at San Filippo Neri Hospital in Italy. The human tissues were obtained and studied in strict adherence to the San Filippo Neri Hospital’s protocol.

### 4.6.6 NanoString nCounter Assay

The NanoString nCounter Human miRNA expression Assay Kit [http://www.nanostring.com](http://www.nanostring.com) was used to profile more than 700 human and human-viral miRNAs in U87 cells treated with Nutlin-3a (10µM) and DMSO. 100ng of total RNA was used as input for nCounter miRNA sample preparation reactions. All sample preparation was performed according to manufacturer’s instructions (NanoString Technologies). Preparation of small RNA samples involves the ligation of a specific DNA tag onto the 3’end of each mature miRNA. These tags are designed to normalize the Tm’s of the miRNAs as well as to provide a unique identification for each miRNA species in the samples. The tagging is accomplished in a multiplexed ligation reaction using reverse-complementary bridge oligonucleotides to direct the ligation of each miRNA to its designated tag. Following the ligation reaction, excess tags and bridges are removed, and the resulting material is hybridized with a panel of miRNA: tag-specific nCounter capture and barcoded reporter probes. Hybridization reactions were performed
according to manufacturer’s instructions with 5µl of the 5-fold diluted sample preparation reaction. All hybridization reactions were incubated at 64 °C for a minimum of 18hr. Hybridized probes were purified using the nCounter Prep Station (NanoString Technologies) following the manufacturer’s instructions to remove excess capture and reporter probes and to immobilize transcript-specific ternary complexes on a sterptavidin-coated cartridge. Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following the manufacturer’s instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high density (600 fields of view) was performed.

4.6.7 Western Blot Analysis
Samples were extracted in 15 mM Tris_Cl, pH 7.5/120 mM NaCl/25 mM KCl/2 mM EGTA/0.1 mM DTT/0.5% Triton X-100/10 mg/ml leupeptin/0.5 mM PMSF. Total protein (50 µg) from each sample was separated on a 4–20% Tris-HCl Criterion precast gel Bio-Rad (cat# 345-0032, Hercules, CA) and transferred to a poly (vinylidene difluoride) filter (Millipore). The filter was blocked in 5% nonfat dry milk, incubated with the specific antibody, washed, and probed with secondary antibody IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology), and developed with enhanced chemiluminescence (Amersham Pharmacia). Immunoblot analyses were performed using the following antibodies: TSC1 (LS-C36590, LifeSpan Bioscience, Inc), p53 (sc-53394, Santa Cruz Biotechnology), Mdm2 (sc-965, Santa Cruz Biotechnology), phospho-Mdm2 (Cat#3521, Cell Signaling), c-MYC (1472-1, Epitomics, Inc), E2F1 (3240-1, Epitomics, Inc), total-Akt (Cat#9272, Cell signaling), phospho-Akt (Cat#4060, Cell Signaling), total-
S6 (Cat#2217, Cell Signaling), phospho-S6 (Cat#2211, Cell Signaling), p21 (sc-817, Santa Cruz Biotechnology), GAPDH (Cat#2118, Cell Signaling).

### 4.6.8 RT-PCR

RNA was isolated from cell lines using Trizol reagent (Invitrogen) as per the manufacturer's protocol. An aliquot of 5 µg RNA was then used for cDNA synthesis using the SuperScript first strand cDNA synthesis kit (Invitrogen). RT-PCRs were carried out using using ABI Prism 7900HT sequence detection systems with Applied Biosystems TaqMan Gene expression assays (p21 (CDKN1A) : Hs01121172_m1; MYC: Hs99999003_m1; E2F1: Hs00153451_m1; TP53: Hs00153349_m1; MDM2: Hs01066938_m1; TSC1: Hs01060648_m1)

### 4.6.9 RNA extraction

Total RNA was extracted using TRIzol Reagent Invitrogen (Cat# 15596-018) following the manufacture’s instruction. Specifically the pellet obtained from 5 X10⁶ cells was lysed 1 ml of TRIzol solution. At the end of the extraction the isolated RNA was dissolved in 35 µl in RNase-free water and incubated for 10 min at 55°C.

### 4.6.10 Chromatin Immunoprecipitation assay

Chromatin immunoprecipitation was performed as described by de Belle et al., 2000 with slight modifications. Cells (5×10⁶) from U87 cells treated with MYC siRNA were fixed in 1% formaldehyde for 10 min at 37°C for chromatin cross-link. Cells were washed with ice-cold 1× PBS, scraped in 1×PBS plus protease inhibitors, and collected by
centrifugation. Cell pellets, resuspended in cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, and 1% SDS] plus protease inhibitors. The probes were sonicated 25x for 30 s with a Bioruptor sonicator (Diagenode) and pelleted. The supernatant was diluted with dilution buffer [17 mmol/L Tris (pH 8.0), 167 mmol/L NaCl, 1.2 mmol/L EDTA, 1.1% (v/v) Triton X-100, 0.01% (w/v) SDS]. DNA-protein complexes were immunoprecipitated using 5 µg of the anti-Myc antibody (Santa Cruz) or with mouse polyclonal IgG control (Zymed). Cross-links in the immunoprecipitated chromatin were reversed by heating with proteinase K at 65°C overnight, and DNA was purified by the MinElute Reaction Cleanup column (Qiagen) and resuspended in water. The purified chromatin was subjected to PCR and the products were analyzed by gel electrophoresis using 2% agarose. The primers are listed in the supplementary information.

4.6.11 In situ hybridization and immunohistochemistry

In situ hybridization (ISH) was carried out on deparaffinized human glioblastoma tissues, which includes a digestion in pepsin (1.3 mg/ml) for 30 minutes. The sequences of the probes containing the six dispersed locked nucleic acid (LNA) modified bases with digoxigenin conjugated to the 5’ end were: miR-25-(5’) cattgcacttgtctcggtctga; miR-32(5’) tattgcacattactaagttgca. The probe cocktail and tissue miRNA were co-denatured at 60°C for 5 minutes, followed by hybridization at 37 °C overnight and a low stringency wash in 0.2X SSC and 2% bovine serum albumin at 4°C for 10 minutes. The probe-target complex was seen due to the action of alkaline phosphatase on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). Negative controls included the use of a probe which should yield a negative result in such tissues. No counterstain
was used, to facilitate co-labeling for TSC1 and p53 proteins. After in situ hybridization for the miRNAs, as previously described (Nuovo et al., 2009), the slides were analyzed for immunohistochemistry using the optimal conditions for TSC1 (1:800, cell conditioning for 30 minutes) and p53 (1:20, cell conditioning for 30 minutes). For the immunohistochemistry, we used the Ultrasensitive Universal Fast Red system from Ventana Medical Systems.

4.6.12 Primer list

**List of primers used for promoter assay of pre- miR-32**

For (-2584): 5’-attgctagecctctgtctacagccaagct-3’

For (-1926): 5’-attgctagegcagtcctaaaatccaatgt-3’

For (-1276): 5’-attgctagttctcctgtctccttta-3’

For (-1110): 5’-attgctagcagtcgctaaaatccacaagt-3’

For (-812): 5’-attgctagctttatttattcctatcctttttta-3’

For (-626): 5’-attgctagcagcttgtgtttttgcagtttaag-3’

For (-326): 5’-attgctagcatactcgtgacacactctttta-3’

For (-83): 5’-attgctagcttcaatgatatctttctttct-3’

Rev (-5): 5’-atttcgagtaaagagatcctgaccactgactc-3’

Rev (-837): 5’-attgctagtgcagtcgatacgatgtaaacaagacacaag -3’

**List of primers used for Chip assay**

pre- miR-32 promoter: For: 5’- cccatctcagttcttttca-3’

Rev 5’-tgccagtatactatggaaccaaga-3’
Cad (positive control): For: 5’-ccagttccccatgttgttgtggtc-3’
Rev 5’-gagagggcatcacagagtgggtgataa-3’

List of primers used for luciferase assay of 3’UTR

3’UTR of TSC1 For: 5’- aattcaggagatgatgtgcaatctgggttt-3’
Rev: 5’- acttctagacaagtgcctgtctccggtaacct-3’

3’UTR of Mdm2 (2137) For: 5-aattctagacctcttgccctgggttaaac-3’
Rev: 5’-acttctagaacttttttccagctgcggttc-3’

3’UTR of Mdm2 (4917) For: 5’-aattctagagacctcaatgagagcaac-3’
Rev: 5’-acttctagatcagcatccaccctaacag-3’

List of primers used for luciferase assay of 3’UTR Mutation

3’UTR of TSC1 Mutation

For: 5’-atggttgctctctttggaactgacctgacatcaatatgt-3’
Rev: 5’-caagattaaatgcctgtcaggttcaagaagcaacat-3’

3’UTR of Mdm2 Mutation (2137)

For: 5’-tcacaaaaactttaaaagaatctcagttgaaggtgga-3’
Rev: 5’-tcacacttaacctttggatgattcttaaatggtgtegtag-3’

3’UTR of Mdm2 Mutation (4917)

For: 5’-tcagggctgtagctagtgtgctcagtcactgacatct-3’
Rev: 5’-agagggtgctgcaagactgactctacgcaggtgag-3’
Figure 4.1 Identification of p53-regulated miRNAs in human GBM
Overview of NanoString assay with U87 cells treated with 10µM Nutlin-3a and with DMSO (B) Western blot in response to Nutlin-3a of p53-silenced (si-p53) and control U87 cells (si-Ctrl). Data are presented as mean ±SD (n=3). (C-D) Tissue microarrays were analyzed by immunohistochemical staining and in situ hybridization. Scale bar; 40µm.

**Figure 4.2 miR-25 and -32 are repressed by p53**

(A) p53 and p21 protein levels in response to p53 induction after the treatment of Nutlin-3a in U87 cells. (B) p21 mRNA levels were determined by qRT-PCR in p53-induced U87 cells. (C), (D) miR-25 and -32 or miR-93 and -106b expression levels in p53-induced U87 cells were measured by qRT-PCR, respectively.
Figure 4.3 p53 and p21 are co-expressed in GBM
(A) Representative cases from 70 glioblastoma specimens in tissue microarrays were analyzed by immunohistochemical staining (p52; red, p21; brown, and co-expression; yellow color). Scale bar; 20µm. (B) Graphs summarizing Chi-Square analysis of immunohistochemical staining results.

Figure 4.4 miR-25 and -32 are overexpressed in human GBM

(A) Representative cases from 60 glioblastoma and 10 normal brain specimens in tissue microarray were analyzed by in situ hybridization (miR-25 and -32). Scale bar; 20µm. (B) Graphs summarizing in situ hybridization staining results. (C) miR-25 and -32 relative expression in glioblastoma tissues (n=57), cell lines (n=7) and normal brain
samples (n=4) was determined by qRT-PCR assay. The relative expression values were used to design box and whisker plots. (D) miR-25 and -32 relative expression in tumor tissues and their adjacent normal tissues was measured by qRT-PCR (n=5).

Figure 4.5 E2F1 and MYC transcriptionally activates miR-25 and -32

(A), (B) E2F1/MYC protein and mRNA levels in p53-activated U87 cells were measured by Western blot and qRT-PCR assays, respectively. (C), (D) Relative expression of the miR-25 and -32 in E2F1/MYC-silenced and overexpressed U87 cells, respectively. (E) Identification of MYC interacting region by using luciferase reporter containing promoter regions of pre-miR-32. (+1) position corresponds to the 5’ terminus of miR-32 hairpin. Putative MYC responsive sequences, E-box, is indicated in red box and miR-32 sequence is in blue. Deletion of E-box is showed in red (X) showing abolition of the promoter activity. EV: Empty Vector. (F) Chip assay after 48hr of MYC knockdown. CAD (Carbamoyl phosphate synthase, Aspartate carbamoyltransferase, Dihydroorotase) was used for as a positive MYC target control, whereas non-specific site served as negative control. (G) Luciferase activity of P3 (with E-box) and P4 (without E-box) reporter
constructs after knockdown of MYC. Luciferase activities were normalized by Renilla luciferase activities. (B-E and G) Data are presented as mean ±SD (n=3) (*, P< 0.05).
Figure 4.6 E2F1 and MYC are transcriptional regulators of miR-25 and -32

(A) E2F1 and MYC mRNA levels in response to knockdown of E2F1 or MYC were measured by qRT-PCR (B) miR-93 and -106b expression levels in E2F1 or MYC-silenced cells, U87. (C) E2F1 and MYC mRNA levels in E2F1 or MYC-overexpressed cells, U87. (D) miR-93 and -106b expression levels in response to overexpression of E2F1 or MYC in U87 cells. (E) C9orf5 and MCM7 mRNA levels in p53-activated U87 cells were determined by qRT-PCR. (F) In E2F1 or MYC-silenced cells, U87, MCM7 and C9orf5 mRNA levels were measured by qRT-PCR. (A-F) Data are presented as mean ± SD (n=3)
Figure 4.7 miR-25 and -32 stabilize p53 to induce cell cycle arrest and inhibit cell proliferation

(A) p53 and p21 protein levels in cells treated with Nutlin-3a in presence of miR-25 and -32. Human U87 cells were transfected with miRNA oligonucleotides (100nM), combined with treatment of Nutlin-3a (10µM). The protein levels of p53 and p21 were measured at every 12 hr after treatment of Nutlin-3a by Western blot assays. Control cells were transfected with scrambled oligonucleotides. (*) indicates p21 protein. (B) Western blot in LNZTA3WT4 cells transfected with miRNAs and treated with cycloheximide (CHX) for the indicated time. Before treated with miRNAs and CHX, LNZTA3WT4 cells were grown without tetracycline for 48 hr to activate p53. Note that the p53 blot for scrambled oligonucleotides (Scr) was exposed longer that that of miR-25 and -32 to achieve equivalent zero points. The p53 protein band intensities were quantified and normalized to GAPDH intensities. (C) MTS assay performed in U87, LNZTA3WT4, and U251 cell lines. Cells were transfected with miR-25, -32, and scrambled sequence (Scr) and were harvested at hours, 24, 48, 72, and 96 after transfection. (D) Flow cytometry analysis in U87, LNZTA3WT4, and U251 cell lines at 48hr after transfection with miR-25, -32, or scrambled oligonucleotides (100nM). (E) Western blot analysis in U87 and LNZTA3WT4 cells transfected with miR-25, 32, or scrambled oligonucleotides (100nM). p53 and p21 protein levels were measured at 48hr after transfection. (B-D) Data are presented as mean ±SD (n=3).
Figure 4.8 miR-25 and-32 stabilize p53 protein to induce cell cycle arrest and inhibit cell proliferation

(A) p53 mRNA levels in U87 cells transfected with miR-25 and-32, or scrambled oligonucleotides (100nM) after treatment of Nutlin-3a. Data are presented as mean ± SD (n=3). (B) p53 protein levels in p53-inducible cells, LNZTA3WT4, with or without tetracycline
Figure 4.9 miR-25 and -32 target MDM2 and TSC1

(A) MDM2 3’UTR contain two predicted miR-25 and -32 binding sites. The sites of target mutagenesis are indicated in red. (B), (C) TSC1 protein levels in LNZTA3WT4 cells transfected with pre-miR-25 and-32, or anti-oligonucleotides (ASOs), respectively. (D) The levels of TSC1 protein and mTOR activity (p-S6) were determined in p53-activated cells, LNZTA3WT4, by Western blot analysis. (E) TSC1 mRNA levels in p53-activated cells, U87 and LNZTA3WT4, were measured by qRT-PCR. Data are presented as mean ±SD (n=3).
Figure 4.10 miR-25 and -32 target MDM2

(A) MDM2 3’UTR contain two predicted miR-25 and -32 binding sites. Reporter constructs, containing a wild-type (left panel) or mutated (right panel) MDM2 3’UTR, were assayed. (B) MDM2, p53, and p21 protein levels in U87 at 24hr after transfection with miR-25 and -32 oligonucleotides (100nM), combined with treatment of Nutlin-3a. (C) MDM2, p53, and p21 protein levels in cells transfected with antisense oligonucleotides (ASOs) (100nM) against miR-25 and -32 in U87 cells. (D) MDM2 and p53 expression levels in U87 cells treated with Nutlin-3a after transfected with miR-25 and 32 were determined by Western blot analysis. (E) MDM2 mRNA expression normalized for GAPDH by qRT-PCR. (F) MDM2 mRNA relative expression in glioblastoma tissues (n=57), cell lines (n=7) and normal brain samples (n=4) was determined by qRT-PCR assay. The relative expression values were used to design box and whisker plots. (G) Graphic of the negative Spearman correlation coefficient ($\rho = -0.629$ or $\rho = -0.597$) corresponding to a decreasing monotonic trend between log of MDM2 mRNA relative expression and log of miR-25 or -32 relative expression ($p < 0.00068$, n=31 or $p < 0.001$, n=31). MRE: miRNA-Response Element. (A and E) Data are presented as mean ±SD (n=3).
Figure 4.11 mir-25 and -32 directly target TSC1

(A) TSC1 3’UTR contains one predicted miR-25 and -32 binding site. The reporter assays were performed three times with essentially identical results. (B) The levels of TSC1 proteins in U87 cells at 48 hr after transfection with miR-25, 32 and scrambled oligonucleotides (100nM). (C) The levels of TSC1 protein in cells transfected with antisense oligonucleotides (ASOs) against miR-25, -32 and scrambled oligonucleotides (100nM). (D) TSC1 expression levels at 48 hrs after knockdown of E2F1 or MYC in U87 cells, respectively. (E) Densitometric analysis showing the effect of knockdown of E2F1 or MYC on TSC1 protein levels. (F) The levels of TSC1 protein and mTOR activity (p-S6) in response to p53 induction in U87 cells were measured by Western blot analysis. (G) TSC1 protein levels and mTOR activity in U87 cells treated with Nutlin-3a and transfected with miR-25/32. (H) Densitometric analysis showing the effect of miR-25/32 on TSC1 protein levels and mTOR activity in Nutlin-3a treated cells, U87. (I) Effects of miR-25 and -32 on mTOR and Akt activities at 24hrs after transfection with miR-25 and -32 in U87 cells. (J) 53 protein levels at 24hrs after knockdown of TSC1. (K) mTOR and Akt activities in TSC1-silenced cells, U87. MRE: miRNA-Response Element. (A, E, and H) Data are presented as mean ±SD (n=3).
Figure 4.12 miR-25 and -32 suppresses tumorigenicity in vivo

(A) Kaplan-Meier survival curve of mice implanted with intracranial U87 ΔEGFR cells treat with PBS, scrambled and miR-25 and -32 (n=6/group). (B) In vivo effect of miR-25/32 on tumor growth of glioblastoma cells transfected with miR-25 and-32 or scrambled oligonucleotides (100nM) by H&E staining. Case I: Coronal section and Case II: Horizontal section. (C) miR-25, -32, TSC1 and p53 expression in tumor tissues using in situ hybridization and immunohistochemlstry. Scale bar; 20µm. (D) qRT-PCR to represent Mdm2 mRNA levels in miRNAs or scrambled tumors. Data are presented as mean ±SD (n=3) (*, P< 0.05). (E) A model for a feedback regulatory loop including miRNAs, MYC, E2F1, and p53.
Figure 4.13 Co-expression assay between p53 and miR-25 or miR-32 in miRNAs-transfected tumors. The small arrow shows the normal mouse brain and the large arrow the human brain cancer. Scale bar; 20µm.
Table 4.1 miRNAs differentially expressed between Nutlin treated cells vs No-Nutlin treated cells

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miRNAs sorted by P value of the univariate test (BRB tools) at the nominal 0.05 level of the univariate.
CHAPTER 5

DISCUSSION
5.1 DISCUSSION

Over the past decade, several genetic pathways involved in tumorigenesis in human cancers have been investigated. It has been shown that a variety of cancers contain a number of common genetic alterations that cause the formation of a tumor. The p53 pathway is frequently inactivated in most human cancers, including multiple myeloma and glioblastoma; with mutations estimated to occur in approximate 50% of all cancers (Nigro et al, 1989). Beside mutation and deletion of TP53, the dysfunction of p53 was mainly attributed to MDM2 mediated effects because p53 levels are regulated by MDM2 which binds to p53 and promotes its degradation (Marine and Lozano, 2010). Therapeutic approaches by inhibition of MDM2 with small molecules, Nutlin-3a, have been shown to induce apoptosis. In addition, it has been reported that p53 alterations were tightly associated with clinical outcome in human cancer (Stuhmer and Bargou, 2006). The possibility that p53 status influences cancer progression was raised in an early study in which p53 mutations were occurred in aggressive cancer, supporting an association between worse survival and the presence of p53 mutations.

The deregulation of miRNAs in cancers with impaired p53-pathway can confer advantages mostly by impaired apoptosis and increased survival due to loss of tumor suppressive or gain of oncogenic potential. In our data, the deregulation of miRNA in MM modulated p53 pathway by targeting a p53 positive regulator and a proapoptotic gene; miR-106b-25 cluster, miR-181a, and miR-32 target PCAF, a p53 positive regulator, to destabilize p53 activity and miR-17-92 cluster target BIM to inhibit apoptosis and promotes cell survival in MM cells. The expression of miR-192, -194, and -215 were
significantly down-regulated during transition from normal PC, via MGUS to intramedullary MM in which TP53 was frequently deleted, suggesting that these miRNAs are associated with prognosis of MM. Recent studies also showed that miRNAs participate in the downstream signaling of the p53 pathway. For example, p53 binds directly to the promoter and transactivates miR-34a gene in response to DNA damage and oncogenic stress, which induce cell cycle arrest, apoptosis or senescence (Chang et al, 2007). Ectopic expression of miR-34 recapitulates the biological effects of p53, inducing growth arrest and apoptosis, through its ability to dampen the expression of pro-proliferation and anti-apoptotic genes such as CCNE2, CDK4, MET and Bcl-2. On the other hand, miRNAs play an important role in p53 pathway by modulating p53 activity directly or indirectly. For instance, miR-29 family regulates p53 activity by targeting p85α which composes the regulatory subunit of phosphatidylinositol-3 kinase (PI3K) (Park et al, 2008). In particular, a recent study revealed that there is a direct feedback circuit of miR-15a/16-1 on TP53 and vice verse; over-expression of miR-15a and 16-1 resulted in significant reduction of p53 and p53 was confirmed to induce these miRNAs through specific promoter binding sites (Fabbri et al, 2011).

In present study, we identified a microRNA/TP53 feedback circuitry that likely underlies the pathogenesis and prognosis in human cancers, multiple myeloma and glioblastoma; the p53-activated miRNAs, miR-192, -194, and -215, are regarded as an amplifier to p53 and p53-dependent mediators of cell cycle arrest and inhibitor of cell growth and migration in multiple myeloma. In glioblastoma multiforme, two miRNAs, miR-25 and -32, are repressed by p53 through p53-dependent respression of E2F1/MYC
and also act as an activator of p53. These findings suggest that miRNAs may be previously unrecognized but integral components of established oncogene and tumor-suppressor networks. Here, there are questions on these feedback regulatory loops; what is important meaning in these feedback systems between TP53 and miRNAs? Why p53-positively or negatively regulated miRNAs show the similar functions in the p53-dependent manner? In accordance with our data, p53-regulated miRNAs commonly activated p53 by targeting common target gene, MDM2, a main negative regulator of p53. However, miR-192, -194, and 215 were modulated by p53 via the direct binding of p53 on the promoter of miRNAs genes, while miR-25 and-32 were indirectly repressed by p53 via p53-dependent, negative regulation of their transcriptional factors, E2F1 and MYC. In spite of this difference in their regulatory mechanism, they are likely to contribute to maintain biological stability in a system in response to external changes and tend to accelerate a process by stabilizing p53. It is related in homeostasis, the property of a system that regulates its internal environment and tends to maintain a stable, constant condition. TP53 is a key tumor suppressor gene that was described as the guardian of the genome due to its role in preventing genome mutation via DNA repair, senescence and apoptosis. In response to the serious stress such as DNA damage and the deregulation of genes, TP53 can play a crucial role in keeping homeostasis by monitoring the abnormal changes and recovering to normal condition. In particular, it could be very important matter that p53 activity is normally maintained with regard to prevent cancer development. In this regard, the p53-regulated miRNAs can play an important role in p53-dependent regulation of cancer progression in human cancer. For example, p53-
activated miRNAs, miR-192, -194, and 215, can be quickly responded to cellular stresses via its direct binding to their promoter and modulate them by restoring p53 activity in Multiple Myeloma. In this process, the direct suppression of MDM2 by these miRNAs can accelerate this rapid regulation in response to genomic disability. Taken together, these miRNAs enhance the role of p53 as a sensor for many cancer-associated stress signals, including DNA damage, telomere depletion, oncogene activation, and hypoxia, which can be translated into effects on cell proliferation, cell death, DNA repair and angiogenesis.

Other p53-regulated miRNAs, miR-25 and -32, are indirectly and negatively regulated by p53, and also induce p53 activity via a feedback loop; there is an intermediary between TP53 and miRNAs, E2F1 or MYC and MDM2, the transcriptional factors of miR-25/32 and a target gene of miRNAs to accumulate p53 levels, respectively. Recently, miRNAs have been identified as a component of E2F1 and MYC networks (Brosh et al, 2008; Lize et al, 2009; Woods et al, 2006). The miR-17-92 cluster is directly up-regulated by MYC and has been implicated as an oncogene in B-cell lymphomas and testicular cancers. The up-regulation of miRNAs by MYC is likely to be an important mechanism contributing to their involvement in cancer. We identified two miRNAs, miR-25 and miR-32, as miRNAs regulated by two transcriptional factors, E2F1, and MYC. Interestingly, unlike the E2F1/MYC-activated miRNAs such as miR-17-92, miR-25 and -32 function as tumor suppressors in glioblastoma. We also provide compelling evidence that p53-repressed miRNAs, miR-25 and -32, capable of playing a crucial role through p53 accumulation by down-regulation of MDM2 as p53-activated miRNAs,
miR-192, -194, and -215 in Multiple Myeloma. Why do MYC/E2F1-activated miR-25 and -32 function as tumor suppressor in glioblastoma? The numerous studies revealed MYC/E2F1 could be act as proto-oncogene and their activation provides strong proliferative stimulus to cell growth. In addition, a mutated version of them was frequently found in many human cancers, which causes to deregulate or over-express MYC/E2F1. Thus, it could be helpful to explain why the levels of miR-25 and -32 expressions are up-regulated in glioblastoma harboring persistent expression of MYC/E2F1. Nevertheless, it was revealed that their activation can also lead to cell death through p53-dependent apoptosis (Matsumura et al, 2003; Pelengaris et al, 2002; Tanaka et al, 2002); MYC activation can elicit p53-dependent apoptosis through induction of the ARF protein that inhibits MDM2-mediated degradation of p53 and stabilizes p53 to cause p53 accumulation and apoptosis (Figure 5.1).

![Figure 5.1 E2F1 and MYC mediate p53-dependent responses](image)

Under the deregulation of E2F1/MYC in cancer, p53 might be needed to maintain genomic stability and tumor prevention by monitoring many biological processes
disturbed by aberrant E2F1/MYC expression. The up-regulation of miR-25 and -32 enhance p53 activity by elevating p53 protein and render the cells more susceptible to p53-dependent stress response. Consequently, aberrant expression of E2F1/MYC can be down-regulated by the elevated p53 in p53-dependent transcriptional repression, as shown in the down-regulation of MYC/E2F1 in response to p53 activation (Figure 4.5). Thus, miR-25 and -32 are likely to function as detectors to monitor the deregulation of E2F1/MYC in cancer, and try to keep balance between p53 and MYC/E2F1 to prevent cancer progression.

Interestingly, in the processing of p53 activation by miR-25 and -32, they not only target a negative regulator of p53, MDM2, but TSC1. TP53 plays an important role as regulator of mTOR (mammalian target of rapamycin) pathway, a critical effector in cell-signaling pathway commonly deregulated in human cancers, by transcriptionally activating TSC2 which serves as a key negative regulator of mTOR with TSC1 (Levine et al, 2006). Mutations in either the TSC1 or TSC2 genes result in the autosomal dominant hamartoma syndrome tuberous sclerosis complex (TSC), which is characterized by benign tumor formation in multiple organs, including the heart, lung, liver and brain. On the other hand, activation of mTOR signaling strongly represses PI3K-Akt signaling pathway via negative feedback loop. Thus, the attenuation of Akt activity can activate p53 by removing the negative regulation normally imposed by MDM2 which is phosphorylated and activated by the Akt pathway. Indeed, the miRNAs-mediated down-regulation of TSC1 increases p53 protein by compensating PI3-Akt signaling, consistent with a fact that mTOR activation in loss of TSC1 inhibit PI3K-Akt pathway and attenuate
Akt activity, and p53 levels are substantially elevated in immunohistochemical staining of TSC tumors. This leads to possibility that miR-25 and -32, at least in part, may contribute to the less aggressive malignancy shown in TSC-related harmartomas patient. Our results provide compelling evidence that p53 inhibit mTOR pathway by activating TSC1. TSC1 activation is caused from the down-regulation of miR-25 and -32 by p53-dependent E2F1/MYC repression; it is plausible that p53-mediated reduction in E2F1/MYC protein prevents the expression of MYC-activated miR-25 and -32. Here, it is worth noting that p53 regulate mTOR pathway with miR-25 and -32-mediated regulation of TSC1. This provides new insights into role of miRNAs in the process of p53-regulated mTOR pathway.

Taken together, through miRNAs/TP53 feedback regulatory loop, p53-regulated miRNAs play a critical role in p53-dependent cellular responses by targeting specific genes involved in cancer progression and p53 tumor suppressor network cross-talks with the miRNA regulation system (Figure 5.2). Further more studies will lead to a better understanding of the function of p53-associated miRNAs involved in pathogenesis in human cancers.
Figure 5.2 Schematic presentation of miRNAs targeting components of the p53 tumor suppressor network
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