MicroRNA REGULATION IN GW/P BODY CELL BIOLOGY, LIPOPOLYSACCHARIDE SIGNALING, AND RHEUMATOID ARTHRITIS

By

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To my mom who has always been supportive, encouraging, and extremely patient. To my husband who has given me love and support. To my mentors who have given me guidance and wisdom.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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MicroRNA (miRNA) are a recently discovered class of small RNA molecules that negatively regulate gene expression by targeting specific messenger RNAs (mRNAs). Once bound to the 3' untranslated region of their target mRNA, miRNA cause the translational repression or degradation of that message. Due to the novelty of miRNA research, the cell biology of miRNA-mediated regulation is not yet well understood, but it is known that miRNA and miRNA-effector proteins such as argonaute-2, GW182, and Rck/p54 all localize to cytoplasmic foci known as GW or P bodies (GWB). To better understand the cell biology of miRNA function, we first examined the relationship between GWB and miRNA. We found that in the absence of mature miRNA, GWB formation was abolished, indicating that miRNA are required for the formation of GWB and may be useful indicators of miRNA activity in cells. Next, we examined the effect of immune stimulation on GWB formation and found that the number and size of GWB increased in response to lipopolysaccharide (LPS) treatment in human monocytes. Furthermore, we found that the expression of three miRNA, miR-146a, miR-155, and miR-132, was concurrently increased in LPS-stimulated cells, and that miR-146a regulated LPS-induced cytokine production. These findings further establish GWB as biomarkers for miRNA activity and demonstrate a role for miRNA regulation in LPS-induced cytokine

production. Finally, we investigated miRNA expression in rheumatoid arthritis (RA) patients compared to healthy controls. Interestingly, the expression of miR-146a, miR-155, miR-132 and miR-16 was significantly increased in the RA patients; however, we found that the increased miR-146a in RA patients did not negatively regulate its target genes. These findings suggest a possible role for defective miRNA regulation in RA pathogenesis, and implicate miRNA expression as a potential marker for RA diagnosis, treatment efficacy, or therapeutic target.

CHAPTER 1
INTRODUCTION

What Are MicroRNAs?

MicroRNAs (miRNAs) are small non-coding RNAs that are 20-22 nucleotides long and were discovered relatively recently. The current known function of miRNAs is primarily in the regulation of certain subsets of messenger RNAs (mRNAs) via hybridization to their 3' untranslated region (UTR) (Fig. 1-1). These miRNAs are often evolutionarily conserved and they regulate their target mRNAs by leading to degradation or repression of translation. Although miRNAs are similar in structure to small interfering RNAs (siRNAs), there are distinctions between the two molecules. While miRNAs are endogenously produced from larger precursor forms, siRNAs are typically exogenously introduced. Most miRNAs bind their target mRNAs by binding partially complementary sites in their 3' UTR (Fig. 1-1), while siRNAs bind their targets at one completely complementary site.

The importance of miRNA regulation to normal cellular functions is becoming increasingly clear as more miRNA targets are discovered. Currently, it is known that miRNA play a role in regulating cellular processes such as apoptosis, differentiation, and the cell cycle. The most recent release of the miRNA sequence database, miRBase (version 10.1), includes 5,395 predicted miRNAs in 12 species of plants and animals and several viruses (1,2). For humans, this database lists 540 predicted miRNA sequences and other bioinformatic predictions indicate that as much as one-third of all mRNAs may be regulated by miRNA (3). Although it was initially believed that miRNAs were encoded in intergenic regions, it is now known that the majority of human miRNA loci are located within the intronic regions of either coding or noncoding transcription units and are transcribed in parallel with other transcripts (4-6).

The identification of miRNA genes is typically achieved using one of three approaches. The first approach is through forward genetics where mutations are identified that produce a certain phenotype. This approach was used to identify the first two miRNAs, lin-4 and let-7 (4,7,8). A second approach is using directional cloning to construct a cDNA library for endogenous small RNAs (9). However, a limitation of this approach is that miRNAs expressed only under certain conditions, or in specific cell types, or at low levels may be difficult to identify. The third approach is bioinformatic predictions. This approach is becoming more popular as advanced prediction programs are developed. Some of the most recent rules of miRNA prediction involve the interaction of miRNA with their target mRNAs and will be discussed later.

Numerous techniques have been developed to detect miRNAs. Northern blotting is widely used, but may not be sensitive enough to detect lowly expressed miRNAs. Quantitative real-time PCR assays have also been developed for the detection of miRNA and have the advantage of increased sensitivity. Additionally, miRNA arrays are being implemented for miRNA expression profiling.

Biogenesis and Maturation of miRNA

First, miRNAs are transcribed from the genome by RNA polymerase II as primary miRNA (pri-miRNA) transcripts that range from hundreds to thousands of nucleotides in length, and like other RNA polymerase II transcripts, are capped and polyadenlyated (10,11). In animals, miRNA maturation is achieved by two main processing steps involving two ribonuclease III (RNase III) enzymes, Drosha and Dicer (Fig. 1-2). Drosha and its partner protein DGCR8 process the nuclear pri-miRNA into ~70 nucleotide precursor miRNA (pre-miRNA) molecule (12-16). The pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin 5/RanGTP which specifically recognizes the structure of pre-miRNA molecules (17-

19). Once in the cytoplasm, pre-miRNA is cleaved by Dicer along with its partner protein TRBP (trans-activator RNA binding protein) into a ~21 nucleotide miRNA duplex similar to siRNA and one strand is selected to be loaded into the RNA-induced silencing complex (RISC) in a dynamic process that is not yet fully understood (20,21). Once loaded into RISC, the miRNA will bind to its target mRNA causing degradation or translational repression (Fig. 1-2).

Recently, an alternative Drosha-independent pathway for miRNA maturation has been reported in *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals (22-24). In this pathway, short hairpin introns are spliced into pre-miRNA mimics referred to as "mirtrons" (pre-miRNAs/introns) that can enter the miRNA-biogenesis pathway without Drosha-mediated cleavage (Fig. 1-2) (22-24). However, mirtrons represent a relatively small population of miRNAs, and the majority of miRNAs are processed by the Drosha-dependent pathway.

Cell Biology of miRNA Pathway

The key components of RISC are the argonaute (Ago) family of proteins. In mammals, there are four Ago proteins (Ago1-4), but only Ago2 is known to function in the miRNA and siRNA pathways. Ago2 has been shown to cleave mRNA targeted by miRNA or siRNA and is known as the catalytic enzyme of RNAi (25,26). In addition, tethering Ago2 to mRNA mimics miRNA-mediated translational repression (27).

In addition to Ago proteins, numerous other proteins are required for miRNA functioning including GW182 and Rck/p54, and these proteins are enriched in discrete cytoplasmic foci known as GW bodies (GWB, Fig. 1-3). In mammals, these foci were discovered in 2002 using an autoimmune serum from a patient with motor and sensory neuropathy (28), and most subsequent GWB reactive sera have been identified from patients with neurological symptoms (33%), Sjögren's syndrome (31%), and various other autoimmune disorders including systemic lupus erythematosus (SLE, 12%), rheumatoid arthritis (7%), and primary biliary cirrhosis (10%)

(29). About the same time period, similar foci were discovered in yeast and referred to as processing bodies (P bodies) or Dcp-containing foci (30-32). Initially named after the identification of the marker protein GW182, GWB are found in all mammalian cells examined to date and were shown to be distinct from other known cytoplasmic organelles such as Golgi complex, endosomes, lysosomes or peroxisomes (28). Immuno-electron microscopy data revealed that GWB vary in size from 100-300 nm in diameter and are composed of 8-10 nm strands or fibrils on which all of the gold labels are clustered (Fig. 1-3 D and E). Also, GWB are devoid of a lipid membrane and vary in size and number during the cell cycle (28,33). The foci are small during early S phase, larger during late S and early G2 phases and absent during mitosis (33). The analysis of GWB during cell proliferation via stimulation of mouse splenocytes and T cells with concanavalin A (ConA) revealed that GWB were larger and more numerous in proliferating cells (33). The GW182 protein was demonstrated to be an essential component of GWB in that knockdown of GW182 results in the disassembly of GWB (33,34).

The involvement of GWB in the miRNA pathway and RNAi is based on the evidence that Ago2, siRNA, miRNA, and mRNAs targeted by miRNA/siRNA all localize to these bodies (35-38). Based on data from several reports, GWB may be useful biomarkers for miRNA or siRNA activity. It was reported in 2006 that miRNA are required for GWB assembly (36). Upon miRNA depletion via knockdown of Drosha or DGCR8 and thus inhibiting the maturation pathway, GWB disassembled (36). However, introduction of siRNA as a surrogate for miRNA enabled GWB to reassemble, indicating that small RNA (either miRNA or siRNA) are required for the formation of these foci (36). A later report demonstrated that transfection of functional siRNA into cells induced larger and more numerous GWB (39). This observation was dependent on the presence of the endogenous targeted mRNA (39). In our most recent study, we show that

lipopolysaccharide (LPS) stimulation of human monocytes results in increased production of miR-146a, miR-132, and miR-155 and increased GWB formation (40)(Pauley et al., submitted). This increase in GWB is dependent on the miRNA-effector proteins Ago2 and Rck/p54, and can be replicated by transfecting pre-miR-146a into the cells indicating that miRNA activity is responsible for the increase in GWB formation (Pauley et al., submitted). Taken together, these studies suggest that GWB may serve as useful biomarkers for miRNA/siRNA activity.

Targeting Specificity of miRNA

Mammalian miRNAs pair to the 3'UTRs of mRNAs. Traditionally, it was thought that the main requirement for miRNA target recognition was the perfect matching of the ~7 nucleotide site called the seed region (Fig. 1-1); however, seed matches do not always confer miRNA-mediated repression of the target mRNA. In 2007, Grimson, et al. combined computational and experimental approaches to uncover five general features of seed matching that increase the accuracy of predicting miRNA:mRNA pairs (41). These features are an AU-rich nucleotide composition near the site, proximity to sites for coexpressed miRNAs (which leads to cooperative action), proximity to residues pairing to miRNA nucleotides 13-16, positioning within the 3' UTR at least 15 nucleotides away from the stop codon, and positioning away from the center of long UTRs (41). Based on these rules, Grimson, et al. developed a new miRNA database (http://www.targetscan.org) to narrow down which miRNA:mRNA target relationships are most promising for experimental follow-up (41). Another popular algorithm used for miRNA target prediction is miRanda (http://microrna.sanger.ac.uk).

Roles of miRNA in Immunology

Regulation of the immune system is vital to preventing many pathogenic disorders including autoimmune disease and cancers, and mammals have developed a complex system of checks and balances for immune regulation in order to maintain self tolerance while allowing

immune responses to foreign pathogens, most of which are not fully understood. Recently, it has become evident that miRNAs play an important role in regulating immune response, as well as immune cell development. Amazingly, a relatively small number of specific miRNAs are coming to light as important regulators of the immune system. Their functions are discussed below and summarized in Table 1-1.

Production of miR-125b, miR-132, miR-146a, and miR-155 in Response to LPS

Three miRNAs, miR-146a, miR-132, and miR-155, were recently found to be upregulated in response to LPS treatment of human monocytes (40). Further analysis of miR-146a induction by LPS, TNF-α, and IL-1β revealed that this induction was NFκB-dependent and the mRNA targets of miR-146a include IL-1 receptor associated kinase (IRAK1) and TNF receptor-associated factor-6 (TRAF6) which are key components in the TLR4 signaling pathway (40). Interestingly, miR-146a was only induced in response to cell-surface TLR (TLR2, TLR4, TLR5) signaling and not intracellular TLR (TLR3, TLR7, TLR9) signaling, indicating that miR-146a plays a role in regulating the innate immune response to bacterial pathogens but not viral pathogens (40).

In mouse macrophages, miR-155 was upregulated by polyriboinosinic:polyribocytidylic acid [poly(I:C)] and IFN-β as well as a number of TLR ligands (42,43). These data suggest that miR-155 is involved in the regulation of both bacterial and viral innate immune responses. Most recently, it was reported that sustained expression of miR-155 in mouse bone marrow cells caused granulocyte/monocyte expansion and these populations of cells were characteristic of myeloid neoplasia (44).

Additionally, miR-125b expression was decreased in response to LPS in mouse macrophages (43). Since miR-125b was shown to be capable of targeting the 3'UTR of TNF-α

mRNA, this miRNA may be down-regulated to allow for the LPS-induced production of TNF-α (43).

The miRNA Let-7i Regulates TLR4

In 2007, it was reported that in human biliary epithelial cells (cholangiocytes), miRNA let-7i regulates TLR4 expression (45). In addition, cholangiocytes infected with *Cryptosporidium parvum*, a parasite that causes intestinal and biliary disease, exhibited decreased expression of let-7i that was associated with an increase in TLR4 expression (45). These data further suggest that miRNA-mediated post-transcriptional regulation is critical for innate immune cell response to microbial infection.

Normal Immune Function, Germinal Center Response, and Generation of Ig Class-Switched Plasma Cells Require miR-155

In addition to its regulatory roles in innate immunity, miR-155 has been shown to be a key factor for normal adaptive immune responses. miR-155 is processed from the non-coding RNA known as bic, which is now known to be pri-miR-155 (46,47). Increased expression of bic/miR-155 is found in activated B and T cells (48,49) as well as in activated macrophages (40,42), and is associated with B cell malignancies (50-52).

In 2007, Rodriguez et al. reported that miR-155 is essential for normal functioning of B and T lymphocytes, as well as dendritic cells (DCs) (53). They found that mice deficient in bic/miR-155 diminished adaptive immune responses. Specifically, the bic/miR-155 deficient mice were unable to develop immunity to *Salmonella typhimurium* after intravenous immunization (53). Further analysis revealed impaired B and T cell functioning as well as defective antigen presentation by DCs (53). These data suggest a regulatory role for miR-155 in the functions of both lymphocytes and DCs.

At the same time, Thai et al. reported that miR-155 regulates germinal center response (54). Initially, they found that germinal center B cells normally upregulate the expression of bic/miR-155 during the course of the germinal center response. Using bic/miR-155 deficient mice, they determined that miR-155 regulates the germinal center response at least in part at the level of cytokine production (54).

Later in 2007, it was reported that miR-155 is also responsible for regulating the generation of immunoglobulin (Ig) class switched plasma cells (55). In this study, B cells lacking miR-155 failed to generate high-affinity IgG1 antibodies (55). A transcription factor, Pu.1, was shown to be targeted by miR-155, and overexpression of Pu.1 leads to the production of fewer IgG1 cells, indicating that miR-155 regulation of Pu.1 may be responsible for the normal generation of Ig class-switched plasma cells (55).

Immune Cell Development Involves miR-150, miR-181a, and miR-223

Several studies have reported the involvement of miRNA in the development of immune cells (56-58). One of the first miRNAs described to have a role in the development of immune cells was miR-181a which was shown to be highly expressed in thymus cells and expressed at a lower level in the heart, lymph nodes, and bone marrow (59,60). In bone marrow-derived B cells, miR-181a expression was shown to decrease during B cell development from the pro B-cell to pre-B cell stage (58). In addition, miR-181a may have a role in regulating lymphocyte development based on evidence that expression of miR-181a in hematopoietic stem and progenitor cells resulted in an increase in $CD19^+$ B cells and a decrease in $CD8^+$ T cells (59). miR-181a has also been shown to modulate T-cell receptor (TCR) signaling, thus affecting the sensitivity of T cells to antigens (60).

Granulopoiesis has been reported to be regulated in part by miR-223 (61,62). Two transcription factors, PU.1 and C/EBP, are reported to control the expression of miR-223 during granulocytic differentiation which in turn controls the development of granulocytes (61,62).

Finally, miR-150, a miRNA that is specifically expressed in mature lymphocytes, was reported to be critical for B cell differentiation (58). miR-150 blocks the development of B cells from the pro-B to the pre-B stage, thereby blocking the production of mature B cells (58). Further studies in mice have shown that miR-150 overexpression results in dramatically impaired B cell development, but normal T cell development (63).

Viruses and miRNAs (miR-32, miR-122, and Viral miRNAs)

Recently, it has become clear that some host miRNAs protect against viral infection, while some viruses have been shown to produce miRNAs of their own that regulate both viral and host genes. In 2005, it was reported that human miR-32 is involved in antiviral defense against primate foamy virus type 1 (PFV1) in human cells via downregulation of at least five different PFV1 mRNAs (64). The downregulation of these viral genes results in slower PFV1 replication.

More recently, Pedersen, et al. reported that IFN-β stimulation of hepatic cells results in the production of at least eight miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448) that have perfect seed complementarity to hepatitis C virus (HCV) mRNAs (65). Additionally, miR-122, a miRNA that was previously shown to be required for HCV replication (66), was significantly reduced in response to IFN-β treatment (65).

These findings demonstrate that the mammalian immune system is utilizing miRNA-mediated gene regulation to combat viral infections. However, it is becoming clear that viruses are also utilizing miRNA in order to evade host immune responses. Kaposi sarcoma-associated herpes virus (KSHV) was found to encode 12 miRNA genes (67-69). Thrombospondin-1, a

strong tumor suppressor and anti-angiogenic factor, was identified as one target of these miRNA suggesting that KSHV-encoded miRNA may contribute directly to pathogenesis (70). It has also been shown that one of these KSHV-miRNA, miR-K12-11, has 100% seed sequence identity with human miR-155, thus likely cross regulates the same endogenous targets as miR-155 (71).

Other viruses, including Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV), are reported to produce their own miRNAs. EBV produces miRNAs during different stages that may contribute to viral maintenance and latency, including miR-BART2 which targets the EBV-DNA polymerase BALF5 (68,72). HCMV-encoded miR-UL112 represses the expression of MHC-class-I-polypeptide-related sequence B (MICB), which is required for natural killer cell-mediated killing of virus-infected cells (73).

Defects of miRNA in Diseases

Countless miRNAs are thought to be involved in cancer based on their expression profiles. The most extensively studied miRNAs involved in cancer are miR-155, let-7, and two miRNA clusters, miR-15a/miR-16-1 and miR-17/miR-18a/miR-19a/miR-20a/miR-19b-1/miR-92-1 (known as the miR-17-92 polycistron).

Cancer and miRNA

In humans, miR-155 is overexpressed in a number of solid tumors, including breast, colon, and lung (74-76). In the lung, angiotensin II Type 1 Receptor is a reported target for miR-155, and high expression levels of miR-155 are correlated with shorter postoperative survival in lung cancer patients (76,77). miR-155 is also overexpressed in several human lymphomas including Hodgkin and B cell lymphomas (51,52,78). Transgenic mice overexpressing miR-155 exhibit B cell proliferation defects and lymphoblastic leukemia (50).

Chronic Lymphocytic Leukemia and miR-15a/miR-16-1

The first evidence that miRNAs were involved in cancer was found by studies on chronic lymphocytic leukemia (CLL). Approximately half of all CLL cases exhibit heterozygous or homozygous deletions of chromosome 13q14.3, but a causative gene in this region remained elusive. In 2002, two clustered miRNAs, miR-15a and miR-16-1 were identified as possible candidates (79). The expression of these miRNA was greatly reduced in the majority of CLL cases examined (79). Further studies revealed that both miR-15a and miR-16-1 posttranscriptionally regulate Bcl2, a potent anti-apoptotic factor, and overexpression of these miRNAs resulted in apoptotic cell death in a leukemia cell line (80). These data demonstrate the significance of miRNA involvement cancer development by regulating apoptosis.

Cancer and miR-17-92

miRNAs may be encoded as clusters transcribed as polycistronic pri-miRNA transcripts. One example of this is the miR-17-92 polycistron which contains 7 miRNAs (miR-17/miR-18a/miR-19a/miR-20a/miR-19b-1/miR-92-1) (81). Overexpression of miR-17-92 has been confirmed in several B cell lymphomas (82) and in various tumors including breast, colon, lung, prostate, and pancreatic endocrine (75). In 2007, Venturini, et al. reported the increased expression of miR-17-92 in certain stages of chronic myeloid leukemia (CML) (83). In a recent study, mice were generated with high expression of miR-17-92 in lymphocytes (84). These mice developed lymphoproliferative disease and autoimmunity and died prematurely (84). This data is consistent with previous reports of lymphoma patients identified with amplifications of the miR-17-92 coding region.

Breast Cancer and Let-7

In 2005, it was reported that the let-7 family of miRNAs negatively regulate the oncogene RAS, and that the low expression of let-7, which leads to high levels of RAS, is

correlated with poor survival of lung cancer (76,85-87). More recently, Yu et al. reported that let-7 regulates the self renewal and tumorigenicity of breast cancer cells (88). Specifically, breast tumor-initiating cells (BT-IC) exhibited markedly reduced levels of the let-7 family miRNAs, and overexpression of let-7 in these cells led to reduced proliferation and mammosphere formation (88).

Altered miRNA expression in different types of cancer is becoming a useful biomarker to help with clinical diagnosis, and may also contribute to the pathogenesis of the disease. As we learn more about the roles of miRNA in cancer development and progression, they may become candidate targets for cancer treatments.

Systemic Lupus Erythematosus and miRNA

In addition to cancer, miRNAs are now reported to play a role in many other diseases including Tourett's Syndrome, Alzheimer's disease, and SLE (89-91). In 2007, a new pathway regulating autoimmunity that involves miRNA was discovered in T lymphocytes as demonstrated in the *sanroque* mouse (92). The latter mouse was originally selected from screening mutant mice derived from the chemical mutagen N-ethyl-N-nitrosourea (ENU), and has been shown to result from a mutation in the gene Roquin that encodes a RING-type ubiquitin ligase. In normal T cells, Roquin regulates the expression of inducible T-cell co-stimulator (ICOS) by promoting the degradation of ICOS mRNA. In *sanroque* mice, the absence of this regulation leads to an accumulation of lymphocytes that is associated with a lupus-like autoimmune syndrome. Yu et al. report that miR-101 is required for the Roquin-mediated degradation of ICOS mRNA (92). Mutations of the miR-101 binding sites introduced into the 3'UTR of ICOS mRNA disrupted the repressive activity of Roquin (92). These findings reveal a critical miRNA-mediated regulatory pathway that prevents lymphocyte accumulation and autoimmunity.

There is emerging evidence for the involvement of miRNA dysregulation in human lupus. A study of 23 SLE patients in China revealed that 16 miRNAs were differentially expressed in SLE patients compared to healthy controls, suggesting that miRNAs may be useful diagnostic markers and may be involved in the pathogenesis of SLE (90). Confirmatory data from other SLE populations as well as defining the potential functional defects are ongoing studies to be reported in the near future.

Components of GW/P Bodies are Targeted in Autoimmune Diseases

In 1994, Satoh, et al. characterized autoantigens of 100/102 and 200 kDa recognized by anti-Su autoantibodies (93). Autoantibodies that immunoprecipitated the 100/102 and 200 kDa proteins were detected in sera of up to 20% of patients with SLE, scleroderma, and overlap syndromes (93). In 2006, Jakymiw et al. reported that anti-Su autoantibodies from human patients with rheumatic diseases and a mouse model of autoimmunity recognize the catalytic enzyme in the RNAi/miRNA pathways, Ago2, as well as Ago1, 3, 4 and Dicer (94). Additionally, by immunofluorescence, the anti-Su autoantibodies were shown to recognize GWB (94).

Recently, an investigation into the clinical and serological features of patients with autoantibodies to GWB revealed that the most common clinical presentations of these patients were neurological symptoms, Sjögren's syndrome, SLE, rheumatoid arthritis and primary biliary cirrhosis (29). The most common autoantigens targeted by these patients were Ge-1/Hedls (58%), GW182 (40%), and Ago2 (16%) and 18% of GWB reactive sera did not react to any of the antigens analyzed indicating that there are other target autoantigens yet to be discovered (29).

The finding that Ge-1 is the most common autoantigen target in GWB was surprising since most studies have focused on GW182 and Ago2. However, in 2005 it was reported that 5% of primary biliary cirrhosis patients have autoantibodies against GWB, and one of these sera

was used to identify the autoantigen Ge-1 (95). Knockdown of Ge-1 resulted in the loss of GWB indicating that Ge-1 is an important component of GWB (95). The function of Ge-1 is not yet fully understood, but some evidence suggests that it may act prior to the 5'-decapping step in mRNA degradation (95). GW182, a 182 kDa protein containing multiple glycine-tryptophan (G-W) repeats and an RNA recognition motif, has also been shown to be required for GWB formation (28,34). Additionally, depletion of GW182 in human cells results in impaired RNAi function (34,96,97).

These data demonstrate an autoimmune response to key components of the RNAi/miRNA pathways which could implicate the miRNA pathway involvement in the induction and production of autoantibody.

Summary

In summary, miRNAs are emerging as important regulators of both innate and adaptive immune responses and development and differentiation of immune cells. Disruption of these critical regulatory molecules can result in the development of cancer and autoimmune diseases. Amazingly, recent work by many groups has unveiled a relatively small number of miRNAs that are involved in regulating the immune system. With the current interests in using siRNA for gene therapy, miRNA targeted therapeutics have a promising future. However, as the miRNA field is still at an early stage, it will be interesting and important to monitor new developments in the next few years whether these are the correct subset of miRNAs critical for immune functions.

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Table 1-1. Important miRNAs in immunology.

miRNA	Function	Target	Reference
hsa-miR-146a	Regulates immune response to bacterial infection	TRAF6 and IRAK1	(40)
hsa-miR-132	Regulates immune response to bacterial infection	Not determined	(40)
hsa-miR-155	Regulates immune response to bacterial/viral infection; Required for normal function of lymphocytes, germinal center response, and generation of Ig class switched plasma cells	PU.1, c-Maf	(40,42-44,48-55)
hsa-miR-125b	Decreased during inflammatory response to allow for TNF-α production	TNF-α	(43)
hsa-let-7i	Regulates innate immune response	TLR4	(45)
hsa-miR-181a	Regulates B/T cell development, modulates TCR signaling	Not determined	(58-60)
hsa-miR-150	Regulates production of mature B cells	Not determined	(58,63)
hsa-miR-223	Regulates granulopoiesis	Not determined	(61,62)
hsa-miR-32	Involved in antiviral defense against PFV1 by downregulating PFV1 mRNAs	PFV1 mRNAs	(64)
hsa-miR-122	Required for HCV replication, reduced in response to IFN-β	Not determined	(65,66)
kshv-miR-K12-11	hsa-miR-155 ortholog	Probably shares endogenous targets with hsa-miR-155	(71)
hcmv-miR-UL112	Inhibits NK-mediated killing of HCMV-infected cells	MICB	(73)
ebv-miR-BART2	Targets EBV-DNA polymerase BALF5 to regulate viral gene expression	BALF5	(68,72)

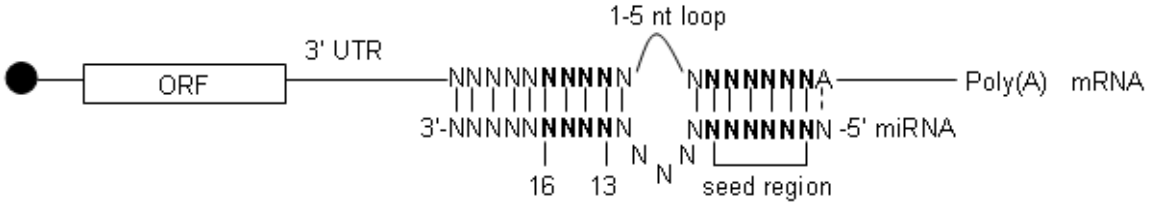

Figure 1-1. Guidelines of miRNA-mRNA interactions. miRNAs bind their target mRNAs by base pairing partially complementary sequences in the 3'UTR. The seed region of miRNA is nucleotides 2-7. This region must be perfectly complementary between the miRNA and mRNA target. At nucleotide position 1, an A residue on the mRNA improves site efficiency, but does not need to base pair with the miRNA nucleotides (indicated by dashed line). The bulges created by mismatched based pairs must be present in the central region of the miRNA-mRNA duplex. In addition, there must be reasonable complementarity between the miRNA-mRNA duplex at nucleotides 13-16 (indicated in bold). These factors improve miRNA binding site efficacy (41).

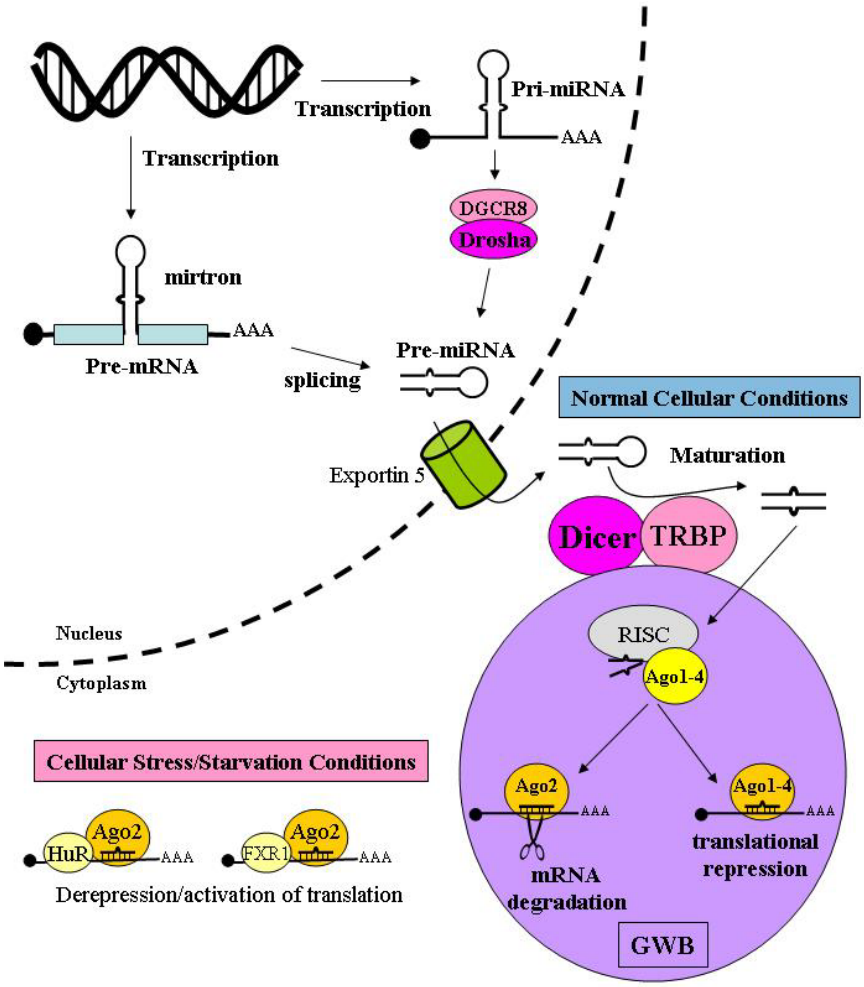

Figure 1-2. Biogenesis and function of miRNAs. miRNAs are transcribed from the genome as long primary transcripts known as primary miRNA (pri-miRNA). The RNase III enzyme Drosha and its partner protein DGCR8 then cleave the pri-miRNA into precursor form (pre-miRNA). In some rare cases, miRNAs can be derived from mRNA splicing of intronic sequences therefore bypassing Drosha/DGCR8 processing. These miRNAs are referred to as "mirtrons." Pre-miRNA is exported by Exportin 5 into the cytoplasm. Once in the cytoplasm, the hairpin loop of the pre-miRNA is cleaved by the second RNase III enzyme Dicer with its partner protein TRBP, and the resulting miRNA duplex is loaded into RISC. Components of RISC, Ago proteins, miRNA and mRNA targets all localize and are highly enriched in cytoplasmic foci known as GW bodies (GWB) or processing bodies (P bodies). The Ago proteins bind the miRNA and target mRNA resulting in the degradation or translation repression of the mRNA. The degree of miRNA-mRNA complementarity dictates whether the mRNA is degraded by endonucleolytic cleavage (perfect complementarity) or translationally repressed (partial complementarity). Under starvation conditions or cellular stress, it has been reported that certain miRNA can activate translation or derepress their target mRNA if certain protein factors (HuR or FXR1) are present (98,99).

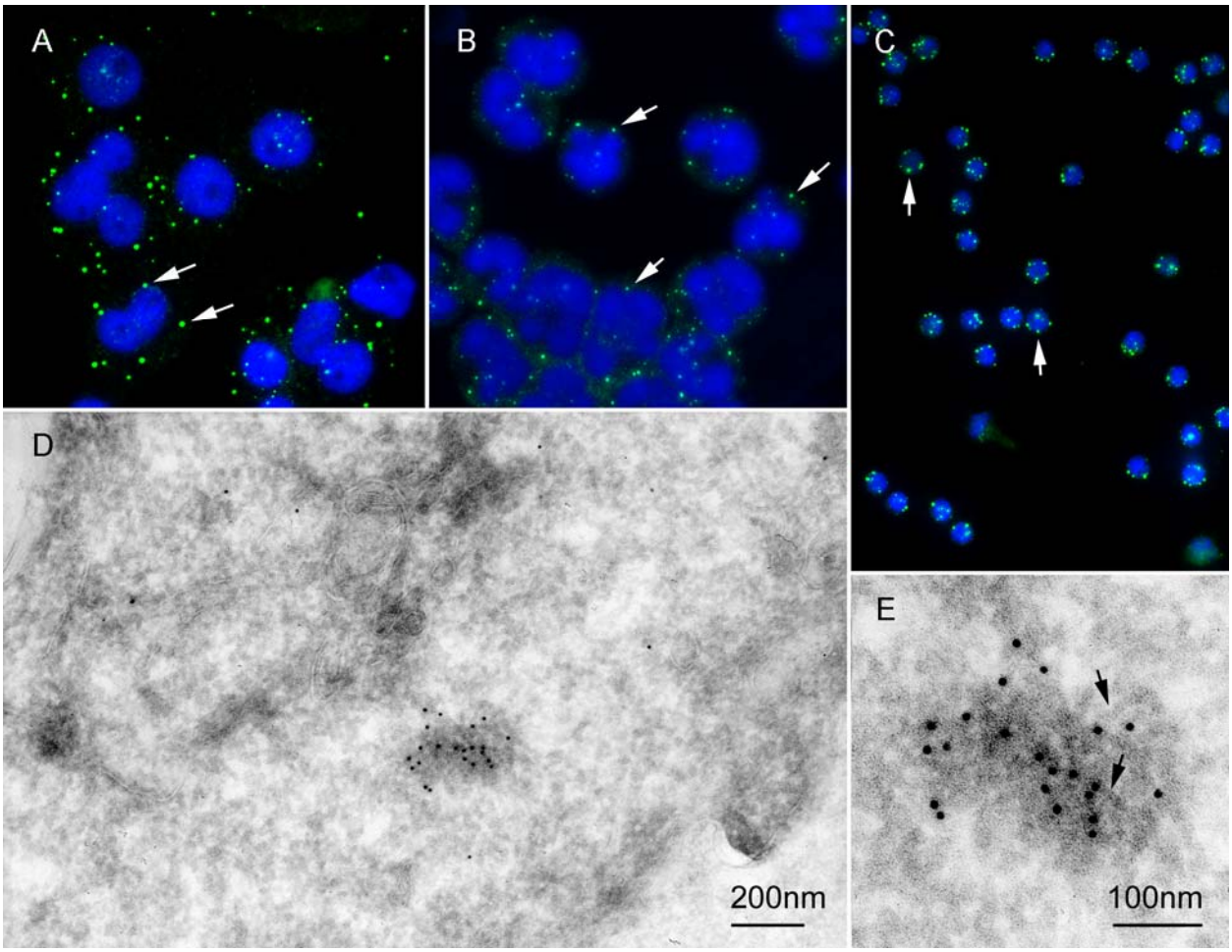

Figure 1-3. GW bodies are present in all mammalian cell types examined to date. (A) THP-1 human monocytic or (B) MOLT-4 cells stained by indirect immunofluorescence with human anti-GWB serum. Arrows indicate examples of GWB. (C) Human peripheral blood mononuclear cells (PBMCs) stained with rabbit antibodies to Rck/p54, another marker for GW/P bodies and a protein required for RNA interference. (D) Immunogold electron microscopy image of GWBs in the cytoplasm of HeLa cells during interphase. Representative gold-labeled cytoplasmic GWBs are structures with diameters varying from 100-300 nm [add reference #33 here]. The gold labels are clustered on electron dense fibrils or strands (arrows), 8 to 10 nm in diameter, which are more obvious in the enlarged image (E). These fibrils appear to form the matrix that the gold decorates.

CHAPTER 2
FORMATION OF GW BODIES IS A CONSEQUENCE OF MICRORNA GENESIS

Introduction

In the past few years, unique cytoplasmic foci have been described in both yeast and mammalian cells that have a role in mRNA processing and degradation. They have been named P bodies in yeast (31,100), and in mammalian cells they have been named Dcp-containing bodies (32,101), GW bodies (GWBs) (28) or mammalian P bodies (35). In this report we will refer to the mammalian cytoplasmic foci as GWBs. GWBs were initially named as such because they represented the cytoplasmic localization of the mRNA binding protein GW182. The GW182 protein has repetitive glycine (G)/tryptophan (W) residues and a canonical RNA recognition motif, and was shown to associate with certain mRNAs in HeLa cells (28). The GW182 protein is a salient feature of GWBs in that reduction of the GW182 protein leads to a decline in the number of GWBs (33). The GW182 protein is conserved among mammalian cells and recently, a protein called AIN-1, with limited sequence similarity to GW182 was identified in *C. elegans* and shown to interact with components of the RISC complex (102). Yeast P bodies and mammalian GWBs are similar in that they are both composed of proteins involved in mRNA degradation, nonsense mediated decay (NMD), and translation (14,31,101,103-108). However, key differences include the observations that there is no protein with significant sequence homology or identity to GW182 in yeast, and GWBs have been linked to RNA interference (RNAi)(34), a pathway that has yet to be described in *S. cerevisiae*.

Interestingly, GWBs appear to be involved in RNAi based on the evidence that Ago2, transfected siRNA, miRNA, and reporter mRNAs targeted by miRNA localize to these bodies (34,35,37,38). Furthermore, the GW182 protein has been shown to play an important functional

role in RNAi (34,96,109). Although GWBs are implicated in the miRNA pathway, the functional requirement of miRNA in GWB formation remains unclear.

These miRNA are small 21-23 nucleotide non-coding RNAs that target specific mRNAs either for degradation or the prevention of its translation into protein via the RNAi pathway (110). Genes encoding miRNA are transcribed by RNA polymerase II to generate the primary miRNA transcript (pri-miRNA), which is capped and polyadenylated (10,11). In the nucleus, a RNase III enzyme known as Drosha, in conjunction with its partner DGCR8 in mammals, or its equivalent Pasha in *Drosophila*, cleave the pri-miRNA transcript producing a ~70-mer hairpin pre-miRNA structure, which is then transported to the cytoplasm via exportin 5 (12-14,16-18,111).

In human cells, studies have shown that knockdown of Drosha results in a sharp decline of pre-miRNA and miRNA accompanied by the accumulation of pri-miRNA (13-16). Furthermore knockdown of DGCR8 with siRNA, also results in a sharp reduction in the amount of pre-miRNAs and hence mature miRNAs (13-15). Taken together, these studies demonstrate the importance of Drosha and DGCR8 in the generation of mature miRNA.

At the start of our study, miRNA localization to GWBs had not yet been reported. As a result, to further clarify the relationship between GWBs and the miRNA pathway, we wanted to demonstrate the presence of miRNA in GWBs and determine whether miRNA were important for the generation of these structures.

Materials and Methods

Short Hairpin RNA Construct

The short hairpin constructs to Drosha, DGCR8, and Narf were obtained from Open Biosystems (Huntsville, AL) and named pDrosha-sh (clone ID V2HS_71783), pDGCR8-sh (V2HS_98193), pDGCR8-sh2 (V2HS_98477), and pNarf-sh (V2HS_50154). Ten additional

unrelated clones were also purchased, and shown to behave similarly as the negative control pNarf-sh.

Cell Culture and Transfections

HeLa CCL2 cells obtained from ATCC were cultured in Dulbecco's Modified of Eagle's Medium containing 10% fetal bovine serum. HeLa cells seeded to approximately 90% confluency in a 6 well plate or 8-chamber slide were transfected with the pDrosha-sh plasmid using Lipofectamine 2000 (Invitrogen) or Arrest-In (OpenBiosystems). For transient transfections, pDrosha-sh was cotransfected with phrGFP maintaining the total DNA amount as per manufacturer's instructions. To generate a homogenous population of Drosha-deficient cells, selection pressure was applied using 2 μg/ml puromycin 2 days after the initial transfection of pDrosha-sh. Ten days after the initial transfection large enough colonies were maintained for further experimentation.

Fluorescent labeled miRNA/siRNA were transfected into HeLa cells or Drosha-deficient cells using Oligofectamine (Invitrogen) as recommended. After 48 hrs of transfection, samples were processed either by IIF, RT-PCR, or Western blot either immediately or at 24 and 48 hr after transfection.

Synthesis of miRNA and siRNA

The miRNA let-7, siRNA to lamin A/C, and siCONTROL RISC-free siRNA were synthesized by Dharmacon Inc., Lafayette, CO. The mature miRNA sequence for let-7 was UGAGGUAGUAGGUUGUGUGGUU. Both the antisense and sense strands, relative to the mature miRNA were synthesized and annealed according to the manufacturer's specified recommendations. The 3'end of the miRNA antisense strand was labeled with Cy3. For siRNA to lamin A/C, the target sequence for lamin A/C was AACUGGACUUCCAGAAGAACA,

which is situated 608-628 nts 3' from the start codon. The 5'end of the siRNA antisense strand was labeled with Cy3.

Indirect Immunofluorescence

HeLa cells were stained as previously described (28). GWBs were detected with human anti-GWB sera (1:2,000 to 1:10,000). Lamin A/C was detected with a mouse monoclonal antibody (Santa Cruz Biotechnology). Drosha was detected with rabbit anti-Drosha antibodies (1:1000). Secondary antibodies used were Alexa Fluor 488 (1:400), Alexa Fluor 568 (1:400), Alexa Fluor 350 (1:100) and Cy5-conjugated goat anti-human IgG (1:100) from Molecular Probes. Glass coverslips were mounted onto the glass slides using either Vectashield Mounting Medium without 4',6-diamidino-2-phenylindole (DAPI) or Vectashield Mounting Medium with no DAPI (VECTOR Laboratories). Fluorescence images were taken with Zeiss Axiovert 200M microscope and a Zeiss AxioCam MRm camera using the 40x 0.75 NA objective. Fixation and permeabilization of cells was done using 3% paraformaldehyde and 0.5% Triton X-100, respectively. Color images were assessed using Adobe Photoshop version 7.

The RNase Protection Assay

Phosphate buffed saline containing complete protease cocktail inhibitors (Roche, Indianapolis, IN) and RNasin inhibitor was added to a 1 ml HeLa cell pellet, containing 10^9 cells and sonicated. The supernatant fractions were then used for immunoprecipitation, which was set up as follows: 200µl protein A Sepharose beads, 20ul anti-GW182 or control serum, 500ul NET2 buffer (150mM NaCl, 5mM EDTA pH 8, 50mM Tris, pH 7.4), 400ul of supernatant. RNA was then isolated from immunoprecipitate by phenol/chloroform extraction and ethanol precipitated. The *mir*Vana™ miRNA probe construction kit (Ambion) was used to synthesize the ^{32}P-labeled *let-7* probe. Probe hybridization and RNase protection was then carried out using the *mir*Vana™ miRNA detection kit (Ambion) according to manufacturer's instructions.

The RT-PCR Assay

RNA was isolated and extracted from HeLa cells using the RNAeasy Mini kit (Qiagen, Mississauga, ON). Reverse transcription was carried out using Omniscript RT (Qiagen). The primers constructed for lamin A/C were as follows: 5' TGGTCAGCCGCGAGGTGT 3' and 5' AGAGGCTGTCGATGCGGATGC 3'. The expected PCR product size was 702bp. The primers constructed for Drosha were: 5' AGTACGCCATAACCAACGAC 3' and 5' GTACTTCCGTTCGATGAACC 3' and the expected PCR product size was 452bp. The primers constructed for human GAPDH were: 5' TGGTATCGTGGAAGGACTCATGAC 3' and 5' ATGCCAGTGAGCTTCCCGTTCAGC 3' and the expected PCR product size was 197bp.
For miRNA RT-PCR, RNA was isolated and extracted from HeLa cells using the *mir*Vana miRNA Isolation kit enrichment procedure for small RNAs (Ambion). The *mir*Vana qRT-PCR miRNA Detection kit and *mir*Vana qRT-PCR miRNA Primer sets were used for miR16, Let7a, miR24 and 5S RNA.

Western Blot Analysis

HeLa cell extract or immunoprecipitated samples were loaded onto a 12% SDS-PAGE gel and transferred using a semi-dry apparatus (BioRad) as described (33). Human anti-GW182 serum was used at a 1:10,000 dilution. The following antibodies/dilutions were also used: rabbit anti-Drosha antibodies (Abcam, Cambridge, MA) 1:500; human anti-lamin A/C antibodies 1:200; mouse anti-tubulin antibodies 1:3,000; rabbit anti-Ago2 antibodies 1:500; rabbit anti-Rck/p54 antibodies 1:500; mouse anti-actin antibodies 1:10,000.

Results and Discussion

To begin, we transfected HeLa cells with Cy3-3'-labeled *let-7* miRNA, to visually observe the intracellular localization of miRNA. We observed that the Cy3-labeled miRNA was located mostly in discrete foci which co-localized with GWBs by indirect immunofluorescence

(IIF) (Fig. 2-1a). Larger foci were also observed that were not GWBs, however based on co-localization with other organelle markers, these larger structures were late endosomes or lysosomes (data not shown). This transfection data is consistent with a recent report showing that HeLa nuclei microinjected with *in vitro* transcribed Cy3-pre-let7 miRNA are found in or adjacent to mammalian P bodies (37). To determine whether *let-7* was naturally found within GWBs, HeLa cell extracts were immunoprecipitated using anti-GW182 antibodies. An RNase protection assay using a ^{32}P-labeled *let-7* probe was then performed to specifically determine whether endogenous *let-7* was localized to the immunoprecipitate. As expected, the immunoprecipitates contained *let-7*, whereas the product immunoprecipitated by normal human serum did not (Fig. 2-1b). This result supported the observations made with the IIF data described above.

Next, we questioned the importance of miRNA in GWB formation and whether the disruption of mature miRNA production would generate a phenotype associated with GWBs. Our approach was based on the published strategy that knockdown of Drosha or DGCR8 resulted in a reduction in the amount of pre-miRNA and mature miRNA after 72 hours (13-15). As a result, HeLa cells were transiently transfected with a plasmid encoding a short hairpin RNA (shRNA) targeting Drosha (pDrosha-sh) to reduce Drosha protein through RNAi. Ten other unrelated plasmids, including one encoding shRNA to nuclear pre-lamin A recognition factor (pNarf-sh), were also transfected into HeLa cells as controls for any nonspecific effect associated with these plasmids. These shRNAs are transcribed in the nucleus and processed by Drosha/DGCR8 in a similar manner to endogenous pri-miRNA. Thus when Drosha/DGCR8 is knocked down by the shRNA-derived siRNA, processing and maturation of newly transcribed shRNAs, as well as endogenous pri-miRNAs, are inhibited resulting in a reduction in these RNA

levels. Since the transfection efficiency using the typical transfecting agent Lipofectamine2000 was ~20-25%, we cotransfected a GFP expression plasmid (phrGFP) such that transfected cells were identifiable. These cells were then stained for GWBs by IIF using human anti-GWB antibodies (28,33,104) and for Drosha protein by rabbit anti-Drosha antibodies. Seventy-two hours after transfection, the majority of cells cotransfected with pDrosha-sh and phrGFP had a clear reduction in number and size of GWBs (Fig. 2-2 iii) and there was a strong correlation with the reduction in Drosha staining (Fig. 2-2, ii). Control cells transfected with phrGFP and pNarf-sh showed a normal distribution of GWBs and Drosha protein (Fig 2-2, v-viii). The Drosha-deficient cells were also stained with an additional P body marker, Dcp1a, which showed an identical phenotype as GWB staining (Fig 2-3).

However, since the transfection efficiency of the plasmid was only ~20% and the number and size of GWB is known to vary during the cell cycle (33), we verified this observation by selecting pDrosha-sh transfected cells by puromycin resistance. After 21 days of selection, we reproduced the phenotype obtained with the transiently transfected HeLa cells, in that the number of GWBs sharply declined in the selected cells (Fig. 2-4b, compare to 2-4a). In a typical experiment, 95% of selected cells completely lacked GWBs with the remaining 5% have residual 1-2 small GWBs. It was confirmed that Drosha protein was substantially reduced in these cells by RNA and protein analysis (Fig 2-5a, 2-5c) and, as predicted, mature miRNA levels were greatly reduced in these cells compared to untreated HeLa (Fig 2-5b). These results suggested that miRNA was central to GWB formation.

We then set out to examine if the introduction of siRNA, as a surrogate for miRNA, could rescue GWB formation. Transfection of the Drosha knockdown cells with lamin A/C siRNA resulted in the reappearance of GWBs in these cells within 48hr (compare Fig. 2-4a, vii

to iv), the localization of siRNA to GWBs (Fig. 2-4d), and a markedly reduced expression of lamin A/C mRNA (Fig. 2-6) and protein (Fig. 2-4c, viii, Fig. 2-4d and Fig. 2-6b). HeLa cells and the Drosha knockdown cells had normal levels of lamin A/C mRNA/protein expression prior to the addition of lamin A/C siRNA (Fig. 2-6a, 2-6b). Transfection of siRNA that cannot be incorporated into RISC ("RISC-free" siRNA) into Drosha-deficient cells failed to rescue GWB formation (Fig 2-7).

To independently substantiate the observed reduction in the number of GWBs seen in Drosha-deficient cells, we transfected HeLa cells with two separate plasmids (pDGCR8-sh, pDGCR8-sh2) encoding short hairpin RNA directed to different sequences in the mRNA for the partner of the Drosha protein, DGCR8. Figure 2-8 shows cells that were transfected with phrGFP alone or co-transfected with both phrGFP and pDGCR8-sh. Interestingly, 72 hours after cotransfection of phrGFP and pDCGR8-sh, most GFP positive transfected cells had a clear reduction in number and size of GWBs (Fig. 2-8, vi), compared to cells transfected with phrGFP alone (Fig. 2-8, iii). Identical results were observed using pDGCR8-sh2. Figure 2-8, vii-ix, shows the reappearance of GWBs in pDGCR8-sh-transfected GWB-deficient cells when they were subsequently transfected with lamin A/C siRNA (compare ix to vi).

Figure 2-9 is a schematic summary of our interpretation of the pathways involved in mRNA targeting by miRNA and their eventual GWB destination. Some of the steps required for pri-miRNA processing to mature miRNA or siRNA are well documented and reviewed elsewhere (110). Based on recently published reports (35,96,112) and on the data presented here, we propose that the initiation of the events leading to the formation of GWBs requires miRNA. We cannot rule out the possibility that miRNA depletion is having a direct or indirect effect on the expression of other components of GWB required for its assembly. However, we showed that

siRNA served as a surrogate for miRNA to restore GWBs and RNAi activity was still functional (Fig 2-6). We further propose that following the initial partnering of the correct mRNA with the Ago2/miRNA complex and perhaps with the assistance of mRNA binding proteins such as GW182, more proteins are recruited leading to the formation of these cytoplasmic foci. The recruited proteins probably include a complex of mRNA degradation associated proteins (95,103,113). Our data is consistent with previous work showing actinomycin D leading to disassembly of GW/P bodies (101) since it is known that miRNA are transcribed by Pol II (11).

In conclusion, GWB formation appears to be miRNA dependent, or most likely Ago2-miRNA and/or Ago2-miRNA/target-mRNA driven, thereby possibly providing a microenvironment for RNAi which would result in either mRNA degradation or repressed mRNA translation. However, we cannot exclude the alternative possibility that the RNAi machinery may be recycled within GWBs, or that the RNAi machinery relocates to GWBs to allow for the ultimate disposal of the targeted mRNA or to prevent its translation. Future studies in examining the requirement of Ago2 and Dicer in GWB formation will be needed to further elucidate the current findings.

This work was published in 2006 in *EMBO Reports* volume 7, issue 9, pages 904-910.

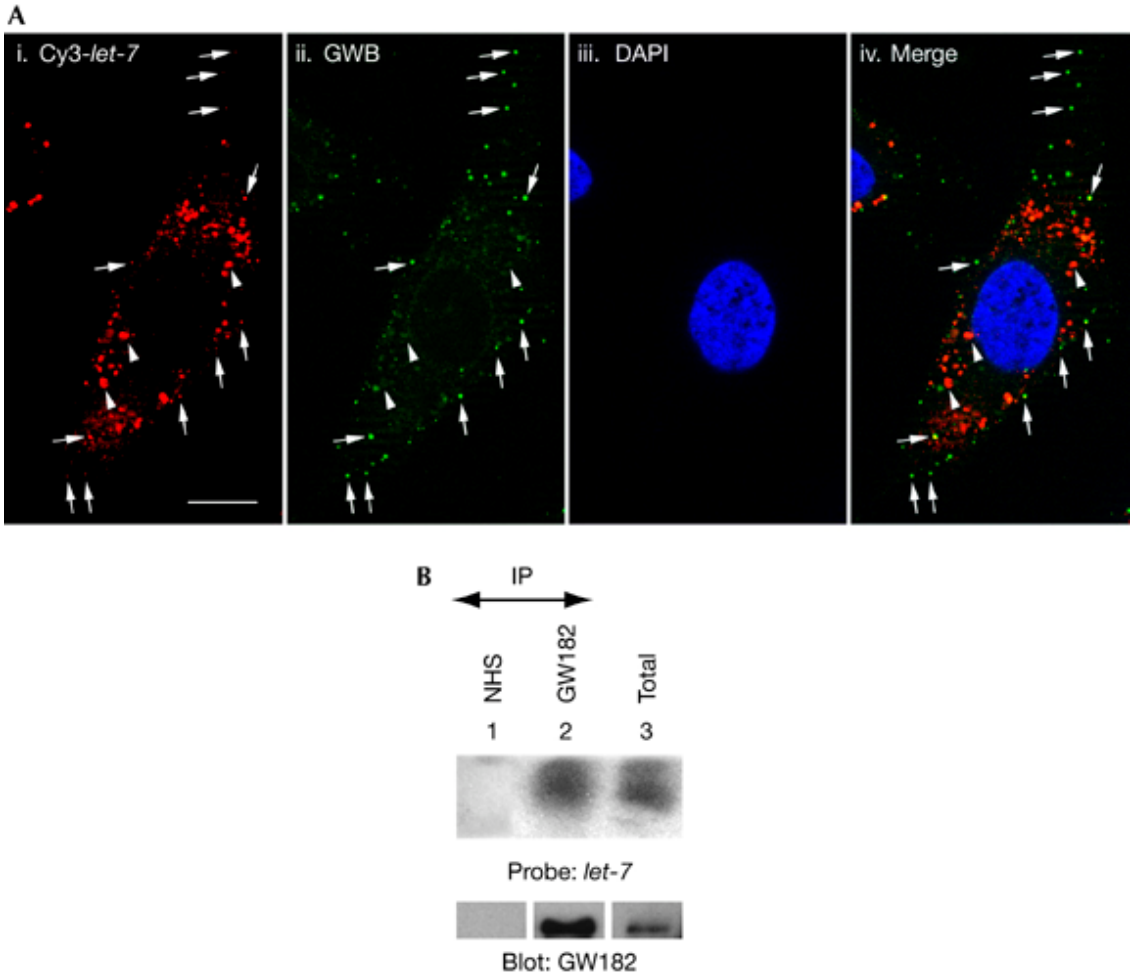

Figure 2-1. MicroRNA is present in GWBs. (A) Transfected let-7 miRNA 3'-labeled with Cy3 was localized to GWBs in HeLa cells 24 hours post-transfection. HeLa cells were transiently transfected with Cy3-3'-let-7 after which they were fixed and stained with human anti-GWB serum. Analysis showed that the miRNA (i, arrows) was found within GWBs (ii, arrows). Nuclei were counterstained by DAPI (iii). Note that miRNA transfected cells often show additional large cytoplasmic aggregates (i, arrowheads) distinct from GWBs (ii, arrows). The merged images are seen in iv. Bar, 10 μm. (B) Biochemical detection of let-7 RNA using an RNase protection assay (upper) and GW182 protein by western blot analysis (lower, lane 2). Lane 1 and 3 are control immunoprecipitate with normal human serum (NHS), and total RNA and protein from HeLa cell extracts, respectively.

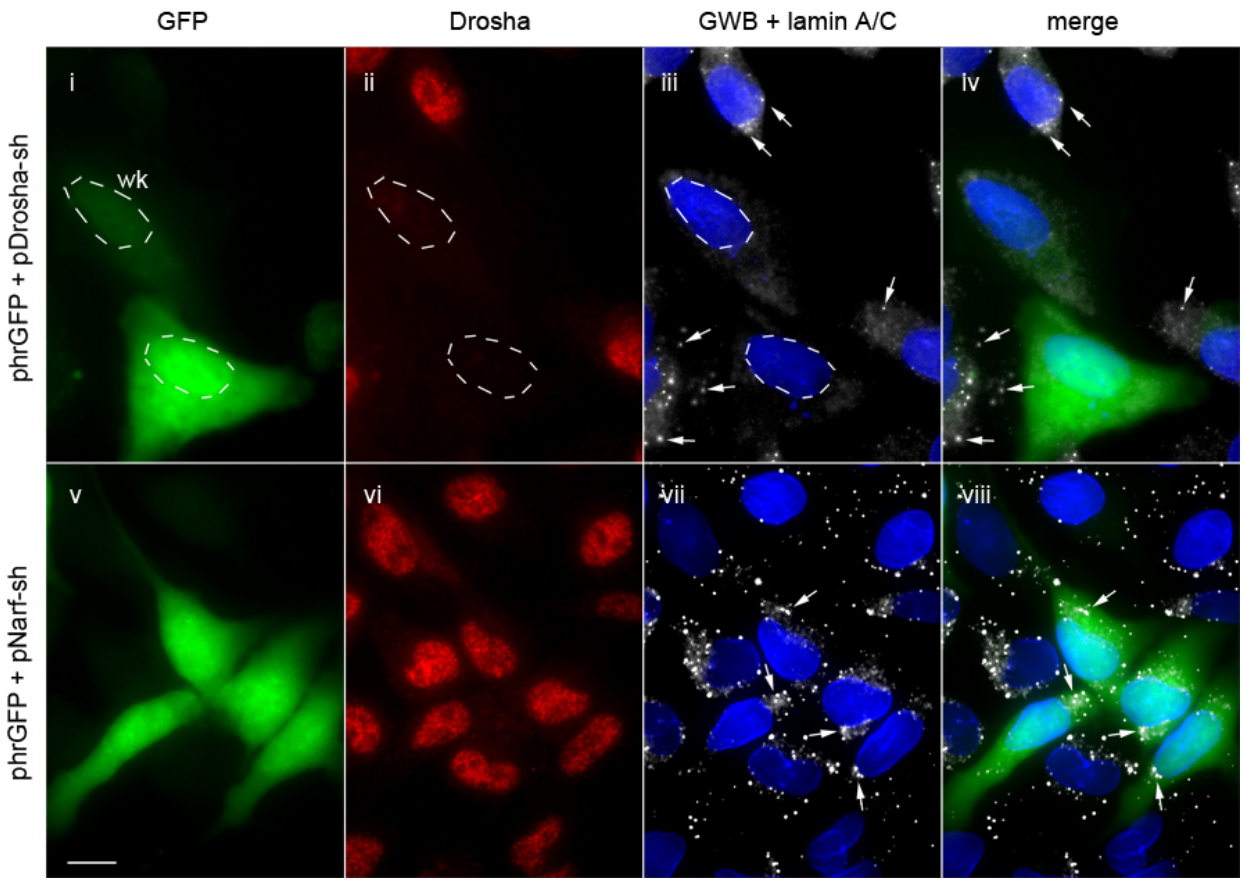

Figure 2-2. Loss of GWB staining in HeLa cells after transient transfection of shRNA plasmid targeting Drosha. A shRNA plasmid targeting Drosha (pDrosha-sh) or unrelated control pre-lamin A recognition factor (pNarf-sh) was co-transfected with the phrGFP plasmid at a 5:1 molar ratio. After 72 hours, cells transfected with pDrosha-sh identified by the co-expression of GFP (i, dashed circular lines, which outline the cell nuclei and also indicate two cells that differ in level of GFP expression; wk, weak GFP signal) showed complete knockdown of Drosha (ii) and virtually complete loss of GWB staining (iii). Untransfected cells had normal GWBs (arrows) and stained positively for Drosha. Control cells transfected with phrGFP and pNarf-sh (v) showed normal expression of Drosha (vi) and GWBs (arrows, vii). Drosha, GWBs, and lamin A/C were detected by rabbit anti-Drosha (red), human anti-GWB (white), and mouse anti-lamin A/C (blue) antibodies as described in Methods. Panels iv and viii, merged images. Bar, 10 μm.

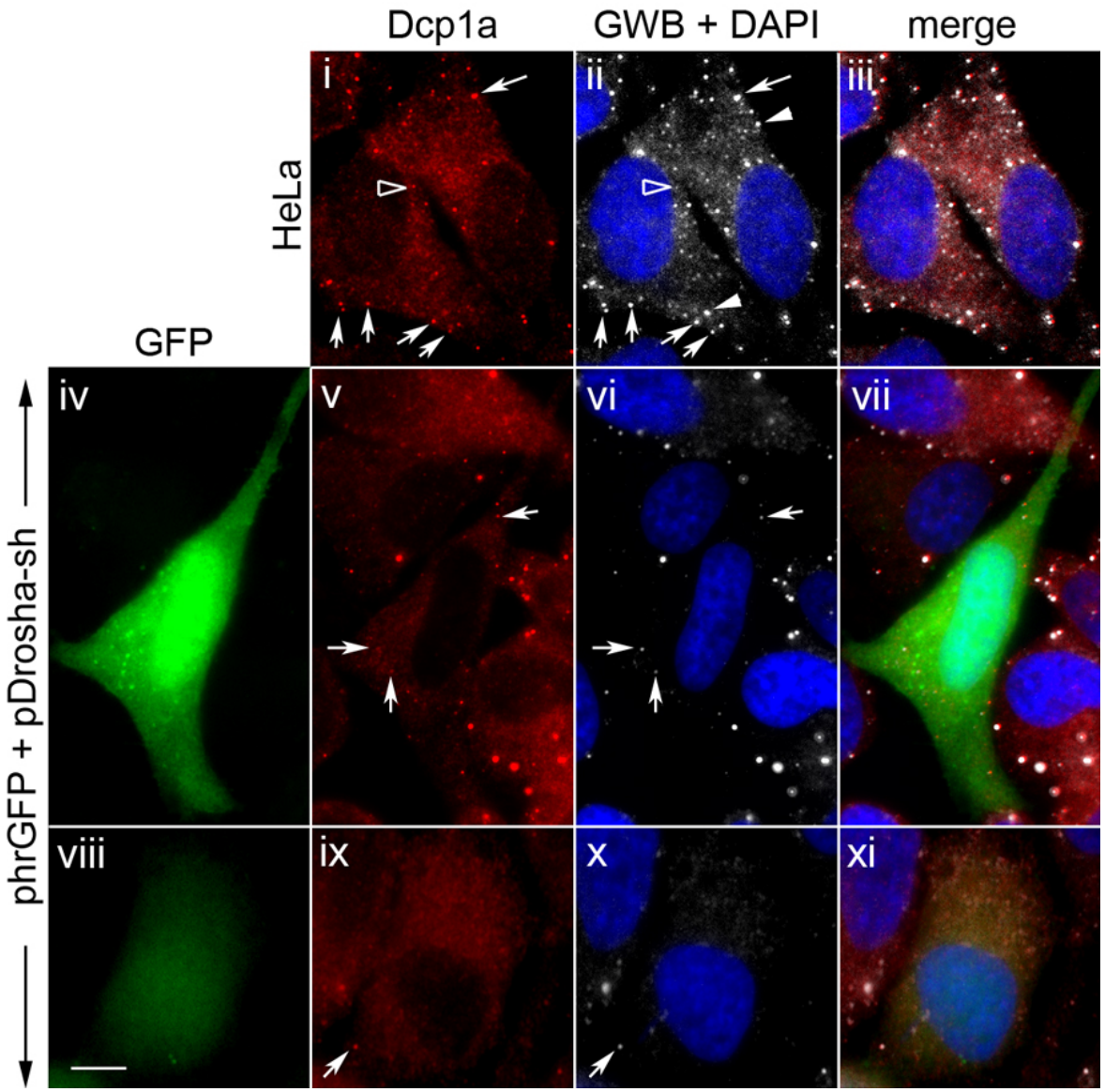

Figure 2-3. Loss of P body marker Dcp1a staining in HeLa cells after transient transfection of shRNA plasmid targeting Drosha. HeLa cells were co-transfected with pDrosha-sh and phrGFP plasmids at a 5:1 molar ratio. After 48 hours, cells transfected with pDrosha-sh identified by the co-expression of GFP showed almost complete loss of GWB staining (vi, x) as well as loss of Dcp1a staining (v, ix) compared to untransfected cells (i, ii). Residual staining of incompletely disassembled GWBs (arrows) in transfected cells are seen in panels vi and x. Panels iv-vii and viii-xi show representative transfected cells with high and low expression of GFP, respectively. Dcp1a and GWBs were detected by rabbit anti-Dcp1a (red) and human anti-GWB (white) serum as described in Methods. Most foci positive for Dcp1a coincide with those positive for GWB staining (arrows); however, there are some foci much stronger in Dcp1a staining (open arrowhead) and others much stronger in GWB staining (arrowheads). Merged images of GFP, Dcp1a (red), GWB (white), and DAPI (blue) are shown in panels iii, vii, and xi. Bar, 10 μm.

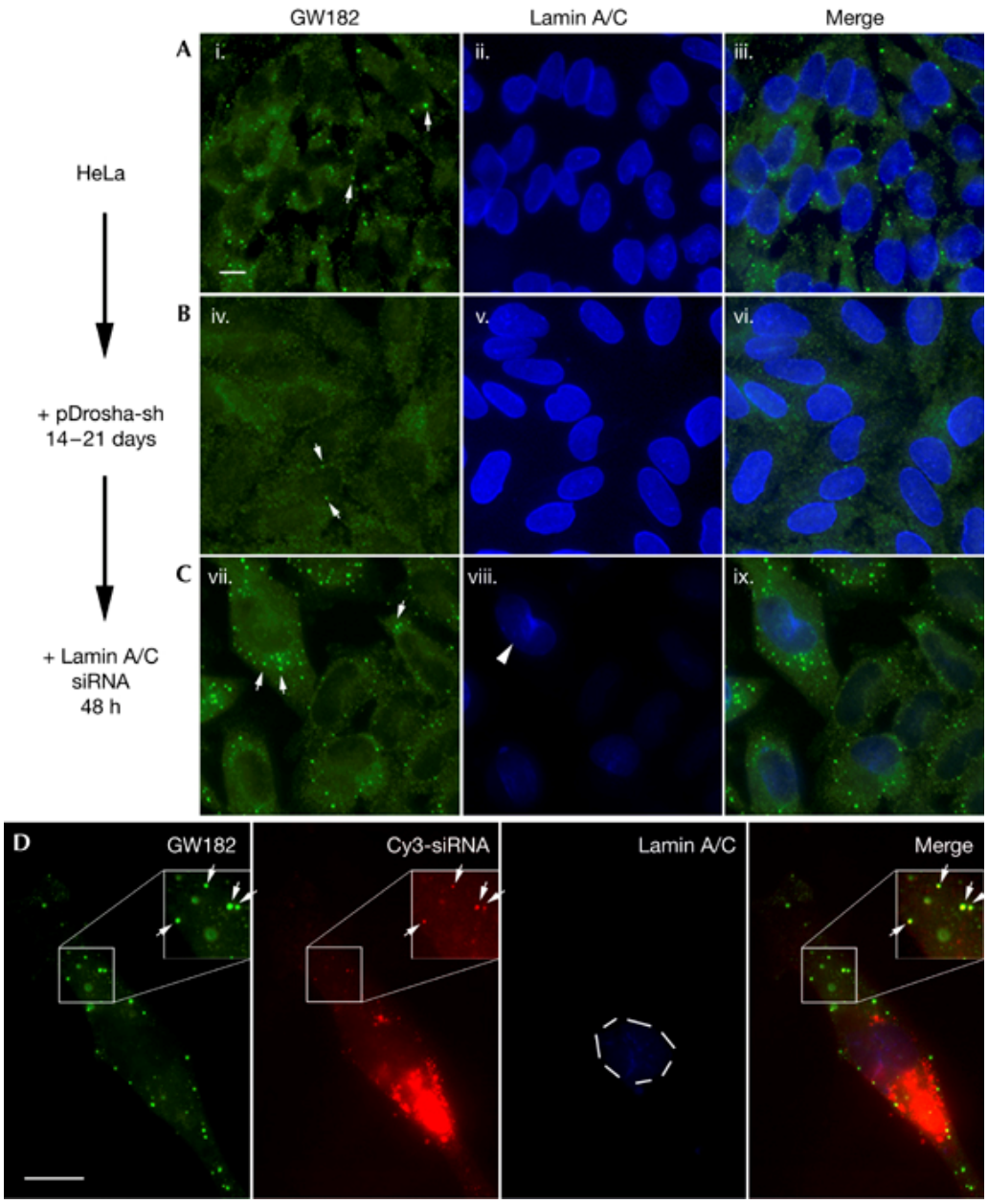

Figure 2-4. Loss of GWB staining in Drosha-deficient HeLa cells and transfection of synthetic siRNAs rescues GWBs. HeLa cells transfected with pDrosha-sh plasmid were allowed to grow in the presence of 2 μg/ml puromycin to select for cells which were later transfected with lamin A/C siRNA. Untreated HeLa (A), Drosha-deficient cells (S4) selected for 21 days (B), and S4 cells 48 hours post transfection with siRNA (C) were co-stained with anti-GW182 antibody and Alexa 488-goat anti-human IgG (green, i, iv, vii) and anti-lamin A/C monoclonal antibody and Alexa 350-goat anti-mouse IgG (blue, ii, v, viii). GWBs (arrows) were readily detected in untreated HeLa cells (i). Most of the GWB staining was lost in S4 cells, but dispersed residual GWB staining was still observable (arrows, iv). Many GWBs were observed in S4 cells after transfection with siRNA (arrows, vii). The knockdown of lamin A/C in S4 cells

transfected with lamin A/C siRNA was effective (viii), with only few cells with detectable lamin A/C (arrowhead). (D**)** Cy3-labeled siRNA for lamin A/C localized to GWBs in transfected S4 cells. The majority of Cy3-siRNA appeared aggregated in the cytoplasm but some were detected in GWBs (arrows). Efficient lamin A/C knockdown was observed and was indicated by the absence of lamin A/C staining in the nucleus (dashed circle). Inserts are 1.5x enlarged and the Cy3 signal is enhanced to show the localization of siRNA to GWBs. Bar, 10 μm.

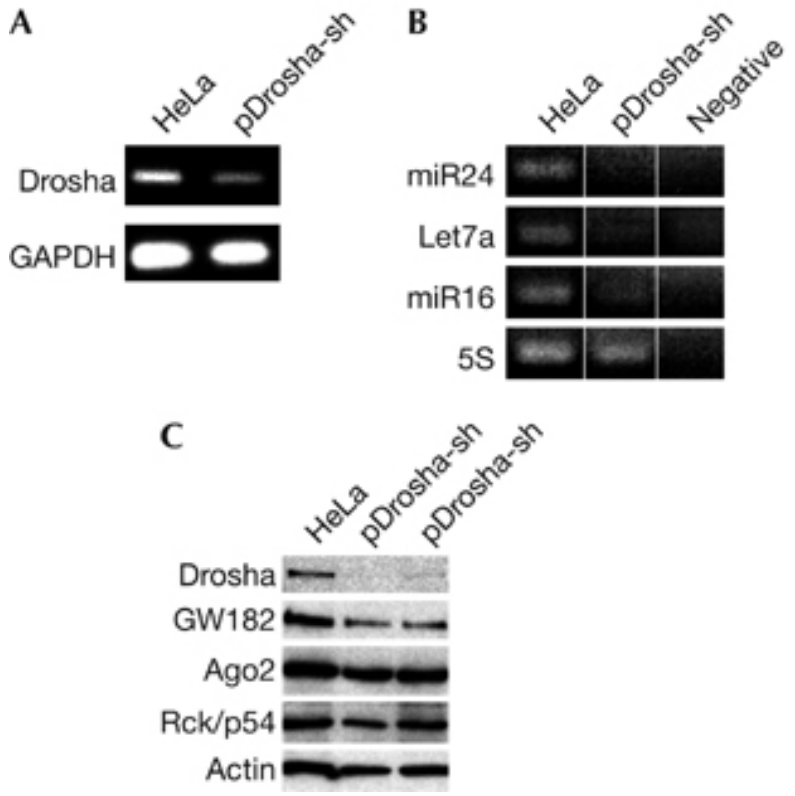

Figure 2-5. Characterization of Drosha deficient cells using RT-PCR and Western blot and demonstration of reduced miRNA levels. (A) Reduction of Drosha mRNA levels demonstrated by RT-PCR comparing untreated HeLa and pDrosha-sh transfected HeLa cells as described in Figure 2-2. GAPDH analysis was included for comparison. (B) Reduction of mature miRNA levels demonstrated by RT-PCR comparing untreated HeLa and pDrosha-sh transfected cells. 5S RNA analysis was included for comparison. (C) Western blot analysis showing a reduction of Drosha protein level in pDrosha-sh transfected cells (data also shown from duplicate experiment) compared to untreated HeLa cells. GW182, Ago2, Rck/p54 and actin levels are shown for comparison.

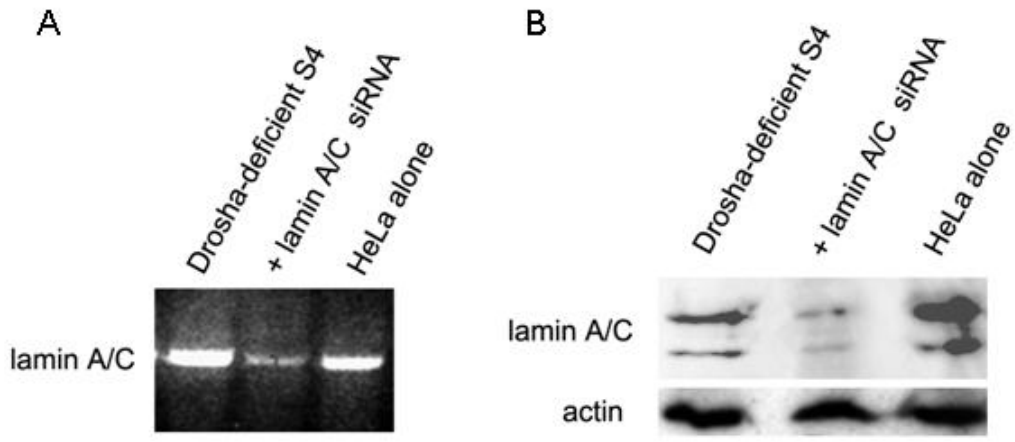

Figure 2-6. Drosha-deficient cells are functionally competent for transfected siRNA-mediated knockdown of target lamin A/C. (A) RT-PCR analysis demonstrating reduction of lamin A/C mRNA levels in Drosha-deficient cells. (B) Western blot analysis of lamin A/C protein levels demonstrating functional RNAi activity when siRNA are transfected into Drosha-deficient cells. Actin levels were monitored to control for sample loading.

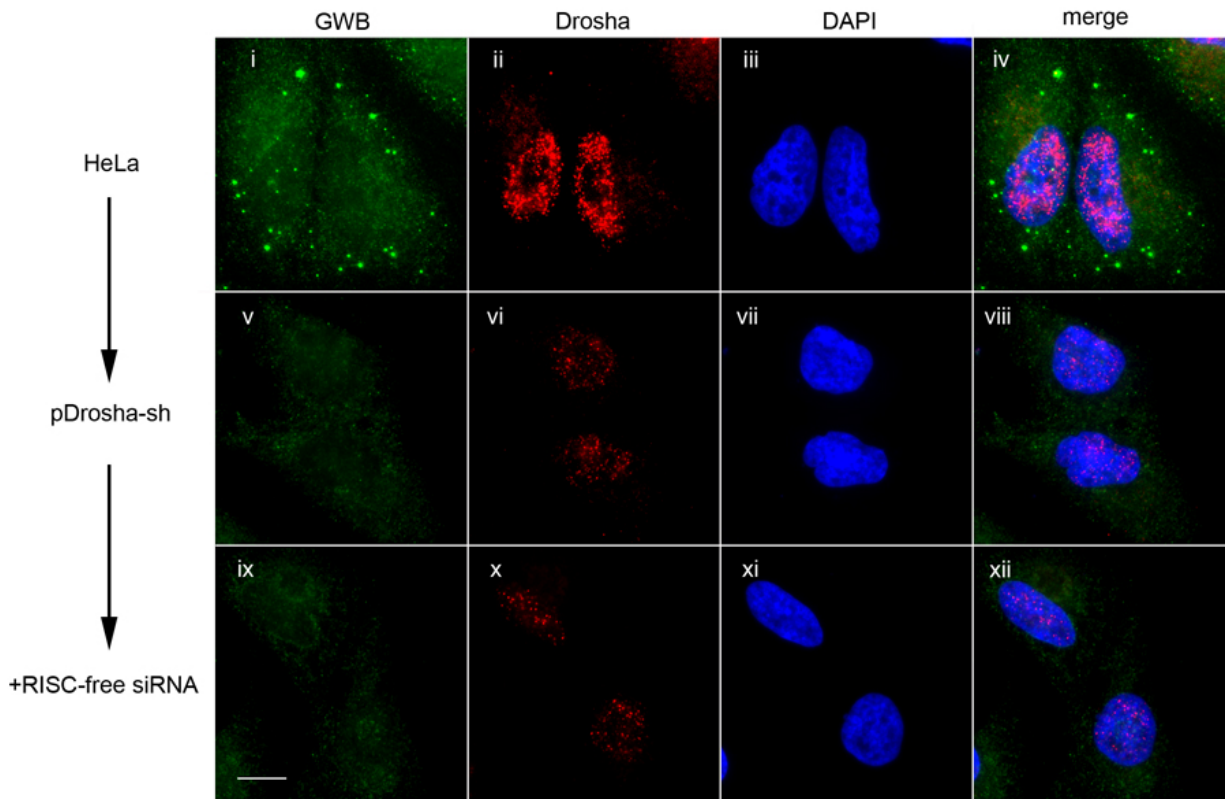

Figure 2-7. Transfection of chemically modified "RISC-free" siRNA did not rescue GWB formation in Drosha-deficient HeLa cells. HeLa cells were transfected with pDrosha-sh plasmid and then subsequently transfected with RISC-free siRNA. Seventy-two hours after the initial transfection, Drosha was drastically reduced in transfected cells (vi) compared to untransfected cells (ii) and there was a loss of GWBs in Drosha-deficient cells (v). Transfection of RISC-free siRNA was unable to rescue GWB formation (ix). Merged images of GWB, Drosha, and DAPI are shown in panels iv, viii, and xii. GWBs and Drosha were detected by human anti-GWB (green) serum and rabbit anti-Drosha (red) antibodies as described in Methods. Bar, 10 μm.

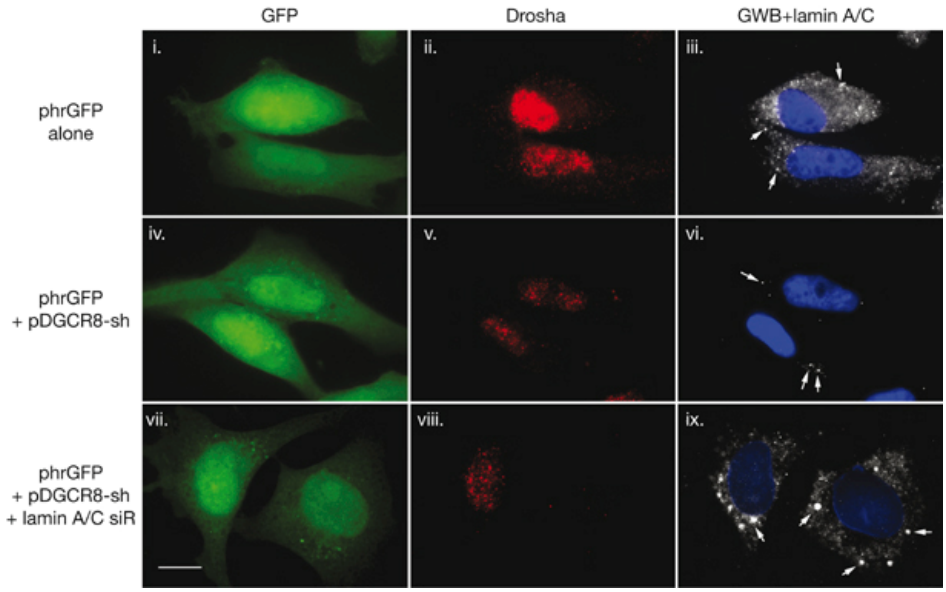

Figure 2-8. Disassembly of GWB in HeLa cells resulting from DGCR8 knockdown and reassembly after siRNA transfection. A shRNA plasmid targeting DGCR8 (pDGCR8-sh) was co-transfected with the phrGFP plasmid at a 5:1 molar ratio. After 72 hours, transfected cells identified by the co-expression of GFP showed almost complete absence of GWB staining, although a few weakly stained GWBs were detectable (arrows, vi). In contrast, GWBs were detected in phrGFP alone transfected cells (arrows, iii). Transfection of siRNA for lamin A/C into DGCR8 knockdown cells resulted in a reappearance of GWBs (arrows, ix) and a reduction in lamin A/C protein in these cells 48 hours post-siRNA transfection. Bar, 10 μm.

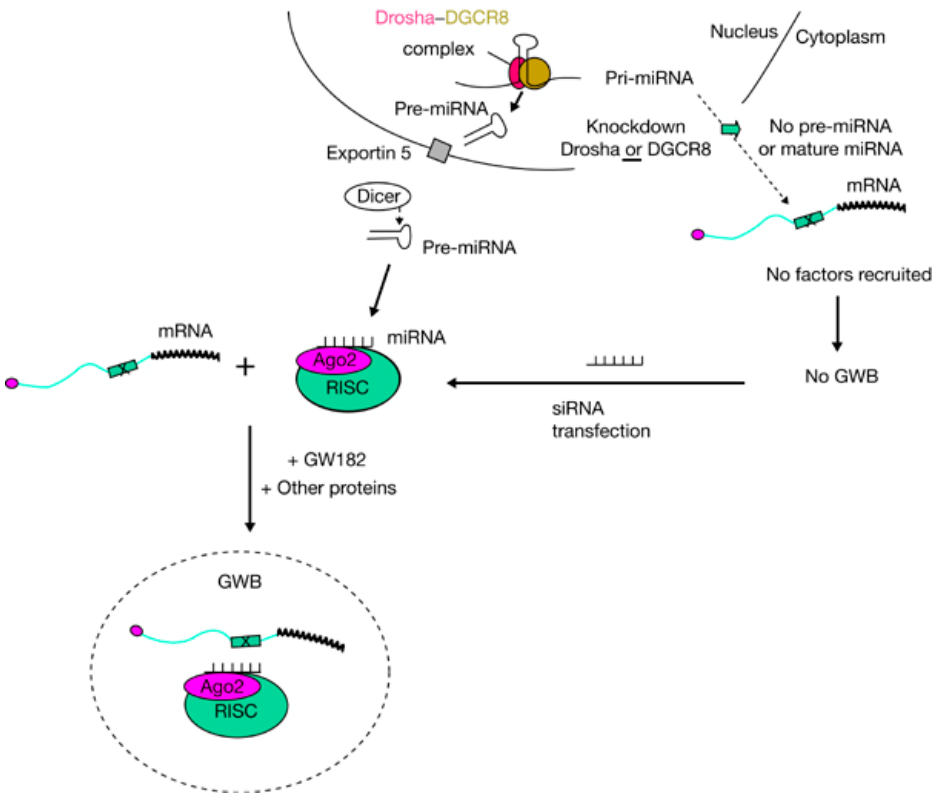

Figure 2-9. The GWB are foci devoted to miRNA processing. In this schematic diagram, pri-miRNA nuclear transcripts are cleaved by Drosha/DGCR8 complex into hairpin pre-miRNA, which are exported to the cytoplasm via exportin 5. In the cytoplasm, Dicer is responsible for cleavage of pre-miRNA generating the mature miRNA. Upon correct interaction and partnering of the miRNA to mRNA containing the miRNA-targeted sequence element "X" in the presence of Ago2/RISC, cytoplasmic foci form upon association of other proteins (see text). However in the absence or low levels of miRNA, as a result of Drosha or DGCR8 protein knockdown, these foci do not form. The transfection of siRNA as a surrogate for miRNA leads to the apparent reassembly of GWBs.

CHAPTER 3
FORMATION OF GW/P BODIES AS A MARKER FOR MICRORNA-MEDIATED REGULATION OF INNATE IMMUNE SIGNALING

Introduction

The cytoplasmic foci known as GW or P bodies, here referred to as GWB, are present in mammalian cells that have a role in mRNA processing and degradation and have recently been linked to the RNA interference (RNAi) and microRNA (miRNA) pathways (114,115). Specifically, it has been shown that small interfering RNA (siRNA) and miRNA localize to GWB (34,36,37). It has also been shown that argonaute 2 (Ago2), the slicing enzyme of RNAi, interacts with a critical GWB protein, GW182, and localizes to GWB (34,38,96,97). In addition, we have shown that the formation of GWB is a consequence of miRNA biogenesis (36), and our latest report demonstrated that siRNA-mediated silencing of endogenous targets results in the induction of GWB assembly (39). Others have also demonstrated the link between RNA-mediated gene silencing and GWB formation (116).

Relatively little is known about the targets of specific miRNA except that most miRNA have specific target sites in the 3' untranslated regions (UTR) of their mRNA targets. AU-rich elements (AREs) are the predominant motifs in the 3'UTR of mRNAs that regulate mRNA stability (117). It was previously thought that AREs were mainly involved in the regulation of relatively few mRNAs such as cytokine and growth factor mRNAs. However, it is now known that ARE mRNAs make up roughly 8% of mRNAs transcribed from the human genome including those involved in cell growth and differentiation, immune responses, signal transduction, and apoptosis (117).

Many cytokine mRNAs, including those for tumor necrosis factor-α (TNF-α), interleukins, interferons, and granulocyte-macrophage colony-stimulating factor (GM-CSF), have very short half-lives, and their degradation is regulated by AREs in their 3'UTR (117).

Since miRNA are known to target sequences in the 3'UTR of mRNAs, it is interesting to postulate that cytokine mRNAs could be regulated by miRNA. In fact, recent studies have reported that miRNA are involved in some ARE-mediated mRNA degradation. In 2005, Jing *et al*. reported that miR-16 targets the ARE in the 3'UTR of TNF-α mRNA and is required for its degradation (118). In 2006, Takahashi *et al.* showed that Dicer is required for ARE-mediated degradation of GM-CSF mRNA, suggesting that mature miRNA play a role in this process (119). Additionally, two recent reports demonstrated that certain miRNA may activate or derepress their target mRNA under specific cellular conditions (98,99). Vasudevan *et al.* reported that miR-369-3 was able to activate translation of TNF-α mRNA, but this activation was not specific to that miRNA-mRNA pair, therefore this phenomenon may be true for other miRNA as well (99).

Further connections between miRNA and the mammalian immune system are becoming more evident with recent publications (120,121). One recent report showed that certain miRNA are upregulated in human monocytes in response to lipopolysaccharide (LPS) (40). LPS is a component of the outer membrane of gram-negative bacteria that activates the production of pro-inflammatory cytokines in monocytes and macrophages. LPS stimulates toll like receptor 4 (TLR4) activating several intracellular signaling cascades that lead to the production of TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1) and other pro-inflammatory cytokines and chemokines (122). Taganov *et al.* reported that LPS stimulation of THP-1 monocytes resulted in increased expression of miR-146a, miR-155 and miR-132, and they showed that TRAF6 and IRAK1, proteins in the TLR4 signaling pathway, were likely targets of miR-146a (40). In addition, miR-155 has also been linked to several other immune functions including germinal

center response (54), normal lymphocyte functions (53), and regulation of immunoglobulin class-switched plasma cells (55) .

Taken together, these recent reports demonstrate an important role for miRNA in normal immune functions. The aim of this study is to investigate the effect of innate immune signaling and related miRNA on GWB since the latter has been implicated in miRNA biogenesis in our earlier report (36).

Material and Methods

Cell Culture and LPS/Cytokine Treatment

THP-1 human monocytes obtained from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum. THP-1 cells were seeded at $5x10^5$ cells per well in a six-well plate and treated with 1 μg/ml LPS from *Salmonella enterica* serotype minnesota (Sigma, St. Louis, MO) unless stated otherwise. After designated treatment time, cells were harvested and washed once in PBS prior to analysis.

Immunofluorescence

THP-1 cells were cytospun onto glass slides at 1,000 rpm for 5 minutes. Cells were fixed in 3% paraformaldehyde for 10 minutes and permeabilized in 0.5% Triton X-100 for 5 minutes. GWB were detected with human anti-GWB sera (1:6000) and rabbit anti-Dcp1a (1:1000). Secondary antibodies used were Alexa Fluor 488 goat anti-human IgG (1:400) and Alexa Fluor 568 goat anti-rabbit IgG (1:400) from Molecular Probes (Carlsbad, CA). Slides were mounted using Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories, Burlingame, CA). Fluorescence images were taken with Zeiss Axiovert 200M microscope and a Zeiss AxioCam MRm camera using the 20x or 40x 0.75 NA objectives. Color

images were assessed using Adobe Photoshop version 7. GWB were counted using Cell-Profiler image analysis software (123).

Western Blots

THP-1 cell extracts were loaded onto a 7.5% SDS-PAGE gel and transferred to nitrocellulose. The following antibodies and dilutions were used: rabbit anti-Ago2 antibodies 1:500; rabbit anti-Dcp1a antibodies 1:1,000; rabbit anti-Rck/p54 antibodies 1:500 (MBL International, Woburn, MA); mouse anti-tubulin antibodies 1:5,000; mouse anti-actin antibodies 1:10,000; rabbit anti-Drosha antibodies 1:500 (Abcam, Cambridge, MA); and rabbit anti-golgin-97 (124) antibodies 1:200. Secondary goat anti-mouse and anti-rabbit antibodies conjugated to horse radish peroxidase (HRP) were used at 1:10,000 dilutions (Southern Biotech, Birmingham, AL). Densitometric analysis of the developed film was performed using Image J software (125). Protein levels were normalized to tubulin and the fold change in protein level between LPS-treated and untreated samples was calculated for each time point.

The qRT-PCR Assay

RNA isolates were prepared using the *mir*Vana miRNA Isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were determined and equal amounts of each RNA sample were used for qRT-PCR. qRT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, and TaqMan MicroRNA Assay primers for human miR-146a , human miR-132, human miR-155 and human let-7a (Applied Biosystems, Foster City, CA). The cycle threshold (Ct) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined and the miRNA expression, relative to untreated controls, was calculated using the $2^{-\Delta\Delta Ct}$ method (126).

Synthesis and Transfection of siRNA and miRNA

siRNAs targeting Rck/p54, Ago2 and Drosha and pre-miR-146a (Ambion) were transfected into THP-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To monitor the transfection efficiency, Cy3-labeled siRNA targeting lamin A/C or Cy3-labeled pre-miR-negative control (Ambion) were transfected into cells in parallel to all transfections, and at least 80% transfection efficiency was achieved. The siRNAs used in this study were all purchased from Dharmacon (Lafayette, CO) and dissolved in 1x Universal buffer (provided by Dharmacon), and the resulting 20μM stock was stored in aliquots at -20°C prior to use. The sense and antisense strand sequences are listed below: hAgo2: 5'-GCA CGG AAG UCC AUC UGA A dTdT-3' and 5'-UUC AGA UGG ACU UCC GUG C dTdT-3' (127); hRck/p54: 5'-GCA GAA ACC CUA UGA GAU UUU-3' and 5'-AAU CUC AUA GGG UUU CUG CUU-3' (127); hDrosha: 5'-CGA GUA GGC UUC GUG ACU U dTdT-3' and 5'-AAG UCA CGA AGC CUA CUC G dTdT-3' (16).

Multiplex Analysis of Cytokines

THP-1 cells were transfected as described above, and then treated with 2 μg/ml LPS for 24 or 48 hours in culture medium. The culture supernatant was then harvested and frozen at -80°C for storage before multiplex analysis. The human cytokine/chemokine LINCOplex premixed kit (Millipore, Billerica, MA) or Beadlyte human 22-plex multi-cytokine detection system (Millipore) were used according to the manufacturer's protocol to quantitatively detect the following human cytokines/chemokines: GMCSF, IFN-γ, IL-1β, IL-6, IL-10, IP-10, IL-12p40, IL-12p70, MCP-1, and TNF-α. Samples were analyzed on a Luminex 200 system (Luminex, Austin, TX).

Results

GWB Increase in Size and Number in Response to LPS Stimulation

Stimulation of human monocytes/macrophages with LPS is a well-characterized model of innate immune signaling (122). To determine if there were any changes in GWB associated with innate immune signaling, we treated THP-1 human monocytes with 1 μg/ml LPS for 0 to 24 hours and monitored GWB by indirect immunofluorescence (Figure 3-1A). Within 4 hours of LPS stimulation the number and size of GWB significantly increased compared to untreated cells. The number of GWB was highest at 8 hours of LPS stimulation, and then declined at 12 and 24 hours. This observation was quantified by using CellProfiler image analysis software to count the number of GWB per cell. As shown in Figure 3-1B, the average number of GWB increased almost 2-fold with 8 hours of LPS treatment compared to untreated cells cultured in parallel (18.6 in LPS-treated versus 10.5 in untreated cells). At each time point examined, there was a significant increase in the number of GWB in LPS-treated cells compared to untreated cells by one-way ANOVA ($P<0.001$). This experiment was repeated more than 10 times with similar results obtained each time over a period of 2 years.

Next, we examined the expression levels of some of the protein components of GWB by Western blot in order to determine if the GWB forming in response to LPS were assembled from *de novo* proteins or pre-existing proteins in the cytoplasm. THP-1 cell extracts were prepared from cells treated with 1 μg/ml LPS for 4, 8, or 24 hours together with paired untreated cells harvested at the corresponding time point (Figure 3-2A). Densitometric analysis of the Western blot data estimated that there were only slight changes in the expression levels of Ago2, Dcp1, and Rck/p54 in LPS treated cells compared to untreated cells (Figure 3-2B). We believe that these modest changes in protein levels do not reflect the significant increase in size and number

of GWB, suggesting that LPS-induced GWB are formed from pre-existing proteins in the cytoplasm rather than entirely from *de novo* proteins.

Upregulation of miR-146a, miR-132, and miR-155 in THP-1 Cells in Response to LPS

Based on the recent work by Taganov *et al.* on the upregulation of certain miRNA in response to LPS (40), and our recent work demonstrating the importance of miRNA for GWB formation (36), we speculated that the LPS-induced increase in the size and number of GWB could be the result of increased miRNA expression in the cells. To test this hypothesis, we first set out to confirm that in our experimental conditions, these miRNA were upregulated in response to LPS. We examined the expression of miR-146a, miR-132, miR-155 and let-7a in THP-1 cells after LPS stimulation by quantitative real time PCR (qRT-PCR). As expected, the expression of let-7a was unaffected by LPS treatment and was used to normalize the expression of miR-146a, miR-155, and miR-132. Fold change in miRNA expression was calculated by comparing 4, 8, 12, and 24 hour LPS treatments to untreated samples cultured in parallel. As shown in Figure 3-3A, miR-146a was increased an average of 19 fold after 4 hours and 28 fold after 8 hours LPS treatment. In addition, miR-132 expression was increased 3.8 fold after 24 hours of LPS treatment (Figure 3-3B) and miR-155 expression was increased 2.6 fold after 8 hours of LPS treatment (Figure 3-3C). These data show that LPS stimulation results in the upregulation of certain miRNA coinciding with an increase in the size and number of GWB, indicating a role for miRNA in the increase of GWB.

The Rck/p54 and Ago2 Proteins are Required for LPS-Induced GWB Assembly

To further demonstrate the connection between LPS-induced miRNA and GWB, we examined the effect of depleting two protein components of GWB that are known to be important for miRNA function, Rck/p54 and Ago2 (127). We used siRNA to knockdown Rck/p54 or Ago2 in THP-1 cells, incubated the cells for 48 hours, and then treated the cells with

LPS and monitored GWB expression by immunofluorescence. Figure 3-4A shows the knockdown of Rck/p54 (~90% reduction) and Ago2 (~70% reduction) under these experimental conditions. As shown in Figure 3-4B, the average number of GWB per cell in the Rck/p54-depleted cells was reduced to 4.7 compared to an average of 9.7 foci per cell in mock-transfected cells prior to LPS treatment. These data are consistent with previous reports demonstrating that Rck/p54 depletion results in the loss of GWB (39,127). In addition, LPS stimulation of Rck/p54 depleted cells did not induce an increase in the number of GWB at all time points examined. At 8 hours, when the maximum increase in GWB after LPS treatment of the mock transfected cells was seen, Rck/p54 depleted cells had an average of 3.1 foci per cell compared to 23.5 foci per cell in mock transfected cells (Figure 3-4B), representing an 87% reduction. Consistent with our previous report (39), in Ago2-depleted cells, the average number of GWB per cell was similar to that of mock-transfected cells prior to LPS stimulation (Figure 3-4B). Like the Rck/p54-depleted cells, there was no increase in the number of GWB in Ago2-depleted cells except after 4 hours of LPS stimulation. We speculate that this initial increase in GWB could be due to incomplete knockdown of Ago2 as shown in Figure 3-4A. Overall, this data demonstrated that Rck/p54 and Ago2 were both required for the LPS-induced increase in GWB assembly, supporting our hypothesis that miRNA is responsible for this phenomenon, therefore in the absence of a functional miRNA pathway, GWB are no longer affected by LPS stimulation.

Transfection of miR-146a Alone Can Induce an Increase in GWB Size and Number

Since LPS has only been shown to induce the expression of a few miRNA (40), and in our experimental system miR-146a was the most affected by LPS treatment, we wanted to demonstrate whether or not the introduction of miR-146a alone into the cells could induce an increase in the size and number of GWB similar to LPS-induced GWB assembly. We transfected precursor miR-146a (pre-miR-146a) at a concentration of 30nM into THP-1 cells and

after 24 hours examined GWB by immunofluorescence. To monitor transfection efficiency, we also transfected the cells with a Cy3-labeled-pre-miR negative control which mimics an endogenous miRNA but has no endogenous target. Figure 3-5A shows the increase in GWB size and number in cells transfected with pre-miR-146a compared to mock transfected cells. As shown in Figure 3-5B, only the cells transfected with pre-miR-146a exhibited greater than 2-fold increase in the average number of GWB per cell similar to the increase seen with 8 hours LPS treatment. This experiment was repeated using 15 nM, 7.5 nM, 3.75 nM, 1.88 nM, and 0.94 nM concentrations of pre-miR-146a, and a similar increase in size and number of GWB can be observed with as little as 1.8 nM pre-miR-146a (data not shown). We also isolated total RNA from the transfected cells and analyzed the mRNA levels of TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor associated kinase (IRAK-1), the putative targets of miR-146a (40), by qRT-PCR. THP-1 cells transfected with pre-miR-146a showed an 85% reduction in the mRNA level of TRAF6 and a 51% reduction in the level IRAK-1 compared to mock transfected cells (Figure 3-5C). These data indicate that miR-146a alone can induce an increase in the size and number of GWB and can negatively regulate endogenous TRAF6 and IRAK-1 at the mRNA level.

MicroRNA Play a Role in the Regulation of THP-1 Cytokine and Chemokine Production

Since we confirmed that miR-146a targets TRAF6 and IRAK-1, we wanted to determine the functional significance of this regulation. To do this, we monitored LPS-induced cytokine and chemokine production in mock transfected cells compared to cells transfected with pre-miR-146a using quantitative multiplex cytokine analysis. The pre-miR-146a and mock transfected cells were treated with 2 μg/ml LPS for 24 hours and the culture supernatants were harvested and analyzed. Five detected cytokines/chemokines (IL-8, IP-10, IL-1β, IL-6 and MCP-1) exhibited decreased production in LPS-induced THP-1 cells transfected with pre-miR-146a compared to

mock transfected cells (Figure 3-6). The production of IL-8, IP-10, and MCP-1 was decreased by approximately 70%, while that of IL-1β and IL-6 was decreased 89% and 92%, respectively (Figure 3-6). In these experiments the production of TNF-α was not affected by transfection of pre-miR146a, but this may be due to the low level of TNF-α produced after 24 hours of LPS treatment (less than 5 pg/ml, data not shown). These data demonstrate that the delivery of miR-146a prior to the addition of LPS inhibits LPS-mediated cytokine/chemokine production; the underlying mechanism may be that miR-146a regulates TRAF6 and IRAK1 and ultimately results in the decreased production of these cytokines and chemokines. To further investigate this mechanism, we performed a similar experiment in TRAF6 or IRAK-1 deficient THP-1 cells and found that different cytokines/chemokines were affected by the knockdown of these proteins (Figure 3-7). This data indicates that miR-146a probably targets a number of proteins in addition to TRAF6 and IRAK-1 and may regulate the production of certain cytokines through other mechanisms not yet understood.

We also wanted to examine LPS-induced cytokine/chemokine production in the absence of miRNA regulation. Since it is unlikely that only one miRNA regulates this process, we depleted all miRNA from THP-1 cells by knocking down Drosha. First we transfected THP-1 cells with siRNA targeting Drosha to inhibit maturation of endogenous miRNA, and then these miRNA-deficient cells were compared to mock transfected cells. Figure 3-8A shows that cells transfected with siRNA targeting Drosha demonstrated at least a 70% reduction in the level of Drosha protein 4 days after transfection. We also examined the amount of miRNA in Drosha-depleted cells 3 and 4 days after transfection. Consistent with previous Drosha knockdown studies (13-16,36), miRNA were depleted to less than 20% of normal levels 4 days after

transfection (Figure 3-8B). Based on these data, subsequent experiments were performed 4 days after transfection.

Next, we examined cytokine and chemokine production in these miRNA-depleted cells compared to mock-transfected cells cultured with or without 2 μg/ml LPS for 48 hours. Multiplex analysis was used to quantitatively detect cytokines in the culture supernatants. Figure 3-8C shows 11 detected cytokines/chemokines in the culture supernatants of mock transfected cells treated with LPS for 48 hours. The percent change in cytokine and chemokine production between mock transfected and Drosha-depleted cells was calculated and is displayed in Figure 3-8D. Interestingly, 8 of the cytokines/chemokines examined (IL-12p70, IL-10, TNF-α, GM-CSF, IP-10, MCP-1, IL-1β, and IL-6) demonstrated at least a 20% increase in production in the supernatants of miRNA-depleted cells, while the other three cytokines examined (IL-8, IFN-γ, and IL-12p40) showed no change or a decrease in production between control cells and miRNA-depleted cells (Figure 3-8D). Certain cytokines, such as IL-12p70, IL-10, TNF-α, and GM-CSF are more affected by miRNA-depletion as demonstrated by a greater than 100% increase in production (Figure 3-8D). These data indicated that while miRNA are not required for the LPS-induced production of the cytokines examined, they play a role in the regulation of cytokine levels in THP-1 cells.

Discussion

Proposed Model for miRNA-Mediated GWB Assembly After LPS Stimulation

Our proposed model for the LPS-induced assembly of GWB via the miRNA pathway is illustrated in Figure 3-9. LPS binds to LPS-binding protein (LBP) which in turn is then transferred to CD14 on the cell surface (step 1) (for review see (122)). LPS-CD14 then interacts with TLR4 and its accessory protein MD-2. TLR4 stimulation by LPS activates signal transduction via MyD88 ultimately phosphorylating IκB (step 2). Phosphorylated IκB is then

released from NFκB and degraded while NFκB translocates to the nucleus and activates the transcription of pro-inflammatory cytokines and miRNA including miR-146a, miR-155, and miR-132 (step 3) (Figure 3-3) (40,122). Among these, miR-146a has the highest fold increase in expression in response to LPS (Figure 3-3) (40).

The miRNA are transcribed as pri-miRNA, which are then processed into pre-miRNA by the Drosha/DGCR8 microprocessor complex (not shown). The pre-miRNA are exported into the cytoplasm by exportin 5 and cleaved by Dicer into mature miRNA duplexes, which are loaded into the Ago2/RISC (RNA-induced silencing complex) complex where they bind their target mRNA (step 4) (20). In the case of miR-146a, TRAF6 and IRAK-1, proteins in the MyD88 signaling pathway, are known target mRNAs (Figure 3-5C) (40).

The miRNA/RISC complex enter the GWB, although it is not clear whether this occurs after the miRNA/RISC complex is formed in the cytoplasm or if the miRNA is loaded into RISC inside GWB (114,115). The influx of miRNA duplexes may result in the rapid 2-fold increase in the number of GWB in the cell within 8 hours of LPS exposure (step 5) described in the current report. We hypothesize that this expansion of GWB in the cytoplasm allows for more efficient inhibition of mRNA targets resulting in the ability of the cell to rapidly regulate signaling cascades and cytokine secretion. The increase in GWB number and size may be due to an accumulation of target mRNAs undergoing degradation by a limited amount of decay factors (128). An alternative interpretation may be that some of the mRNAs are being degraded independently of miRNA via a GWB mediated pathway, but this seems unlikely based on the evidence shown in this report of miRNA involvement.

The target mRNAs are translationally repressed (or degraded) resulting in a reduction of protein expression (step 6). TRAF6 or IRAK-1 regulation by miR-146a results in the subsequent

blockage of NFκB activation leading to the downregulation of inflammatory cytokine production (Figure 3-6). Depletion of miRNA or GWB by knocking down Drosha, Rck/p54 and Ago2 results in a lack of regulation after exposure to LPS (step 7). This lack of regulation leads to overproduction of inflammatory cytokines (IL-6 shown as example) and may affect other monocyte functions (Figure 3-8D).

Observation of GWB as Biomarkers for miRNA Activity

Our previous report showed that miRNA biogenesis is required for GWB assembly under normal cellular conditions (36). Specifically, we knocked down Drosha or DGCR8 with short hairpin RNA plasmids in order to stop miRNA maturation at the pri-miRNA level. After mature miRNA were depleted in the cytoplasm, we observed that GWB were disassembled. Our data showed that mature miRNA are required for normal GWB assembly, and exogenously introduced siRNA can act as a surrogate for endogenous miRNA to reassemble GWB (36). Our most recent study also showed that GWB assembly is also linked to siRNA activity (39). We reported that the transfection of siRNA into mammalian cells led to an increase in number and size of GWB, and that this siRNA-induced GWB assembly was dependent on the presence of an endogenous target mRNA (39). Also, like the data reported here for LPS-induced GWB, Rck/p54 and Ago2 proteins were found to be required for the siRNA-mediated increase in GWB (39). These reports show that GWB can serve as biomarkers for miRNA and siRNA activity. In this report, we describe a more natural condition with inducible miRNA from LPS stimulation of TLR4 demonstrating a mechanistic relationship between GWB and miRNA activity (Figure 3-9). Our data extend the current knowledge of GWB and their relationship to innate immune signaling, and are consistent with previous reports demonstrating GWB as biomarkers for miRNA and/or siRNA activity (36,39).

Depletion of miRNA Affects Cytokine and Chemokine Production

Our finding that the production of some cytokines and chemokines was increased in cells depleted of miRNA suggests that miRNA play a role in the regulation of these functions. Interestingly, the production of IL-6, IP-10, MCP-1, IL-10, IL-1β, IL-12p70, TNF-α, and GM-CSF were increased by miRNA depletion indicating that the production of these cytokines is regulated by miRNA (Table 3-1). However, two of the cytokines examined (IFN-γ, and IL-12p40) exhibited a decrease in production in response to miRNA depletion and IL-8 was not affected by miRNA depletion (Table 3-1). This suggests that miRNA may positively regulate the production of these cytokines (99), or that miRNA are not involved in their regulation. However, upon transfection of pre-miR-146a, only five of these cytokines/chemokines exhibited decreased production (IL-6, IP-10, IL-8, MCP-1, and IL-1β, Table 3-1). This discrepancy in data could be due to the fact that some cytokines are directly regulated by miRNA or indirectly regulated by miRNA through other signaling pathways. On the other hand, the data show that major cytokines and chemokines (IL-6, IP-10, IL-8, MCP-1) induced by LPS in THP-1 cells do appear to be regulated by the major induced miRNA miR-146a (Table 3-1).

The regulation of TNF-α and GM-CSF mRNA by miRNA is consistent with previous reports (118,119). These reports demonstrated that miR-16 was able to target the ARE in the 3' UTR of TNF-α mRNA (118), and that Dicer, an RNase III enzyme that cleaves pre-miRNA into mature miRNA, is required for the ARE-mediated degradation of GM-CSF mRNA (118,119). This led us to investigate whether the other cytokines that were increased in response to miRNA depletion contained predicted miRNA binding sites or ARE in their mRNA. The number of predicted miRNA binding sites in each cytokine mRNA were compiled from two different miRNA databases. As shown in Table 3-1, the number of predicted miRNA binding sites for a particular mRNA can vary greatly between the different databases demonstrating the current

limitation in these predictions. However, our data indicating miRNA-regulation of cytokine production is overall consistent with the predicted miRNA binding sites of these cytokines. There are some exceptions where our data indicate miRNA regulation while there are no predicted miRNA binding sites and vice versa. These exceptions may be due to current limitations in accurately predicting miRNA binding sites in mRNA.

In summary, our report has shown that GWB can serve as biomarkers for miRNA activity during innate immune signaling. We have demonstrated that miRNA may play an important role in regulating human monocyte functions such as cytokine and chemokine production. These findings may be clinically significant for patients suffering from autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, and rheumatoid arthritis that are driven by the overproduction of inflammatory cytokines, and further investigations are needed to evaluate the therapeutic potential of these findings.

This work has been submitted for publication in 2008 in *Molecular Biology of the Cell*.

Table 3-1. Potential miRNA regulation of cytokine/chemokine mRNAs in THP-1 cells.

LPS induced Cytokine/Chemokine[a]	ARE in 3'-UTR[b]	Number of TargetScan predicted miRNA binding sites[d]	Number of miRBase predicted miRNA binding sites[f]	Decreased production in pre-miR-146a transfected cells[g]	Increased production in miRNA depleted cells[h]
IL-6	yes	2	12	yes	yes
IP-10	no	2	17	yes	yes
IL-8	yes	0	10	yes	no
MCP-1	yes[c]	2	20	yes	yes
IL-12p40	yes	2	15	not detected	no
IFN-γ	yes	1	19	not detected	no
IL-10	yes	5	16	not detected	yes
IL-1β	yes	0	19	yes	yes
IL-12p70	yes	1	18	not detected	yes
TNF-α	yes	0[e]	19	no	yes
GM-CSF	yes	2	13	not detected	yes

[a]Listed in order of level of production as shown in Figure 7C. [b]Compiled using the Human AU-Rich Element-Containing mRNA Database (ARED) 3.0 (129). [c]From reference (130). [d]Conserved miRNA binding sites compiled from TargetScan 4.0 (www.targetscan.org) (41). [e]No predicted conserved miRNA binding sites, 1 miRNA reported to target TNF-α mRNA (118). [f]Compiled from miRBase version 4 (http://microrna.sanger.ac.uk) (131). [g]Data from Figure 6. [h]Data from Fig 7D.

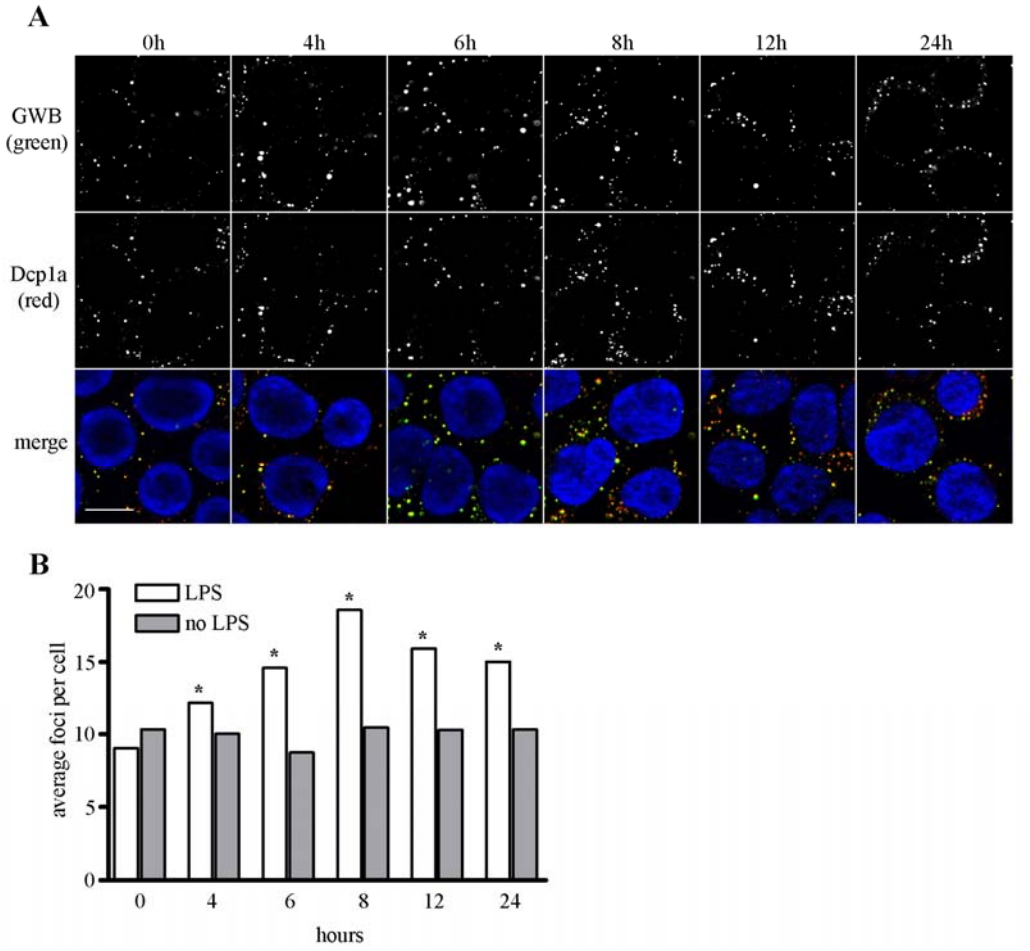

Figure 3-1. Assembly of GWB is induced by LPS. (A) THP-1 cells treated with 1 μg/ml LPS showed a time-dependent increase in the number and size of GWB. After LPS treatment, cells were fixed and costained with human anti-GWB serum (green) and rabbit anti-Dcp1a (red). Nuclei were counterstained by 4,6-diamidino-2-phenylindole (DAPI, blue). Images were acquired at 400x original magnification. Images shown are representative of ten independent experiments. Bar, 10 μm. (B) Bar graph showing representative data from the CellProfiler image analysis software used to quantitate the average number of foci per cell in untreated cells or LPS-treated cells for 0 to 24 hours after LPS incubation (n>150 cells analyzed for each treatment). Asterisks (*) indicate $P<0.001$ compared to untreated control as determined by one-way ANOVA.

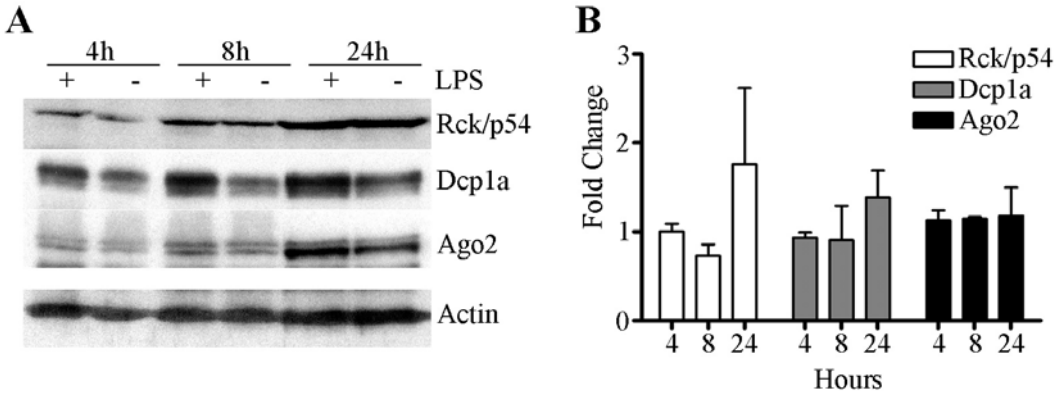

Figure 3-2. Expression of GWB protein components in THP-1 cells unaffected by LPS treatment. (A) THP-1 cell extracts from LPS treated (+) or untreated (-) cells for each time point were compared using Western blot detection of Rck/p54, Dcp1a, Ago2 and actin, which is shown as a loading control. Representative data shown from 3 independent experiments. (B) Quantitative analysis of 3 independent western blots. Rck/p54, Dcp1a and Ago2 protein levels were first normalized to tubulin protein, and then the fold change between LPS-treated and untreated samples was calculated. Average plus SD is shown.

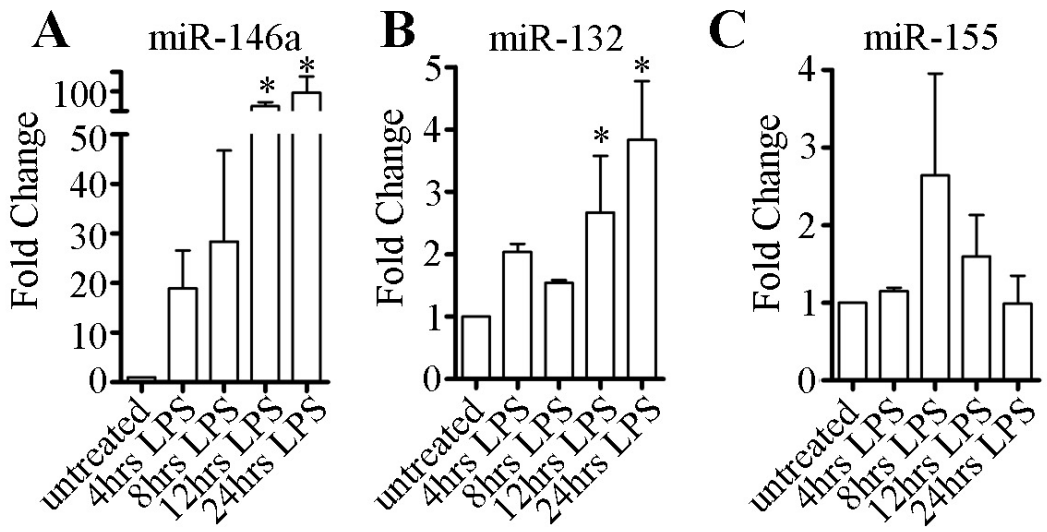

Figure 3-3. Stimulation with LPS induces upregulation of miR-146a, miR-132, and miR-155. THP-1 cells were stimulated with 1 μg/ml LPS for 4, 8, 12, or 24 hours or left untreated. RNA was isolated from cells and qRT-PCR analysis was performed as described in Methods. Fold change miR-146a (A), miR-132 (B), and miR-155 (C) from LPS-treated cells compared to untreated cells cultured in parallel was calculated after normalization to let-7a expression. Average plus SD shown, n=3 independent experiments. Asterisks (*) indicate $P<0.05$ compared to untreated controls.

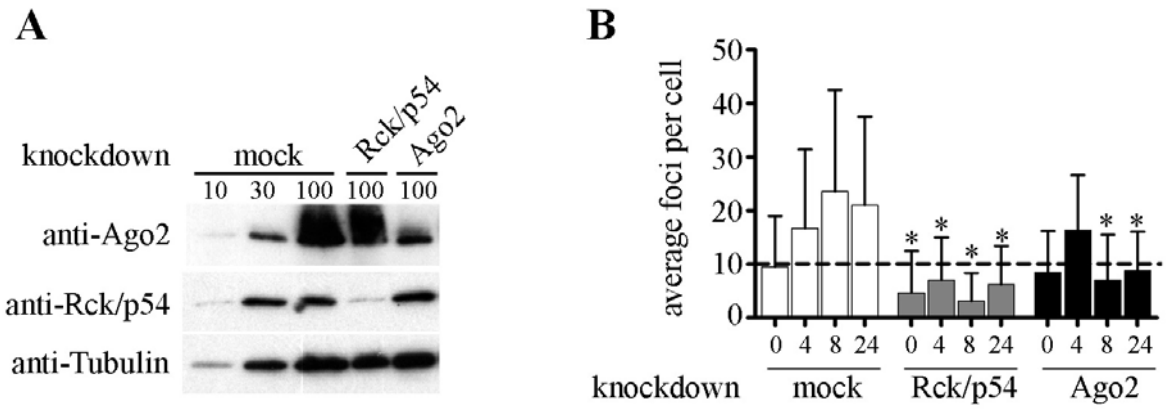

Figure 3-4. Depletion of Rck/p54 and Ago2 results in loss of LPS-induced GWB assembly. (A) Reduction in protein levels of Rck/p54 and Ago2 demonstrated by using Western blot to compare mock transfected cells or cells transfected with siRNA targeting Rck/p54 or Ago2. Tubulin levels were included as a loading control. Cells were harvested 48 hours after transfection. For the mock transfected cells, 10, 30, and 100 indicate percent input of cell lysate. (B) LPS-induced GWB assembly was abolished by knockdown of Rck/p54 or Ago2. THP-1 cells were transfected with siRNA targeting Rck/p54 or Ago2, or mock transfected, and then 48 hours later the cells were stimulated with 1 μg/ml LPS for 0, 4, 8, or 24 hours. GWB were monitored by immunofluorescence and counted using CellProfiler image analysis software. Average plus SD is shown, n>250 cells analyzed in two independent experiments. Asterisks (*) indicate $P<0.001$ compared to corresponding time point for mock transfected cells as determined by one-way ANOVA. Dashed line indicates average foci per cell in normal untreated cells.

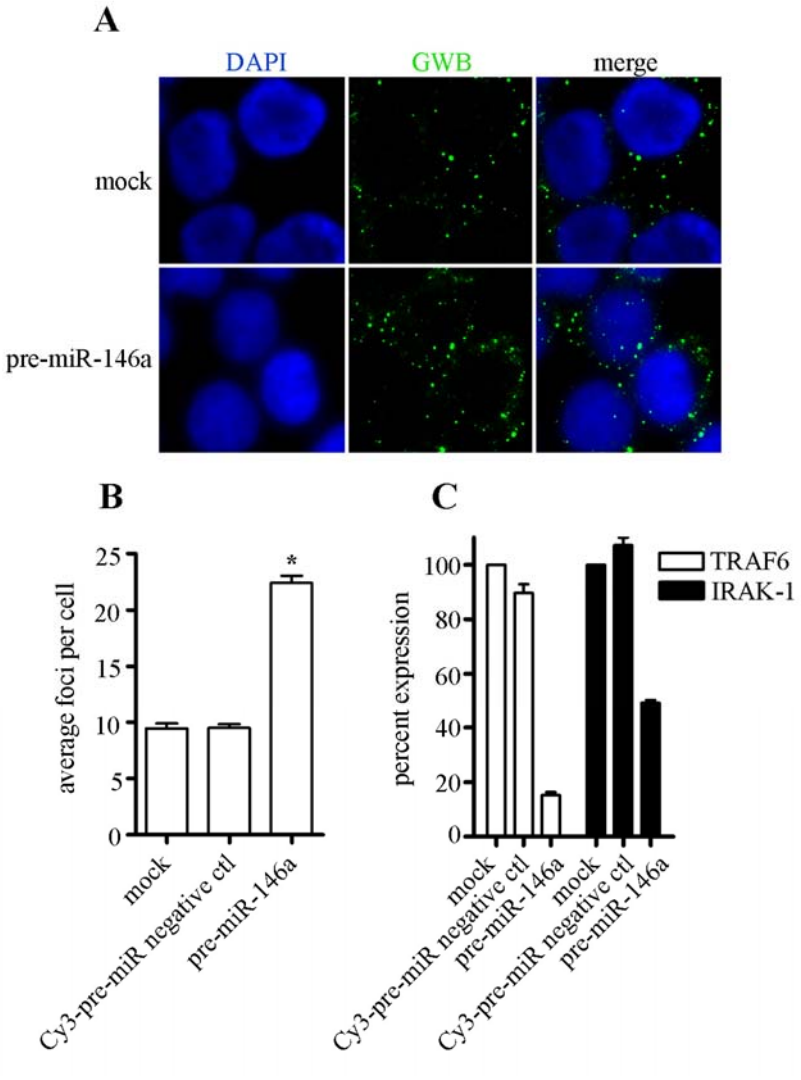

Figure 3-5. Transfection of miR-146a alone can induce formation of GWB and repress TRAF6 and IRAK-1 mRNA. THP-1 cells were transfected with 30nM pre-miR-146a or Cy3-labeled pre-miR negative control and incubated for 24 hours and compared to mock transfected cells. (A) GWB staining showed cells transfected with pre-miR-146a exhibited an increase in the number of GWB compared to mock and negative control (not shown). (B) GWB were counted using CellProfiler image analysis software. Average and SEM is shown, n>300 cells analyzed. Asterisks (*) indicate $P<0.001$ compared to mock transfected cells as determined by one-way ANOVA. (C) Total RNA was isolated from pre-miR-146a, negative control pre-miR, and mock transfected cells 48 hours after transfection and analyzed for TRAF6 and IRAK-1 expression by qRT-PCR as described in Methods. Average plus SD is shown, n=2 independent experiments.

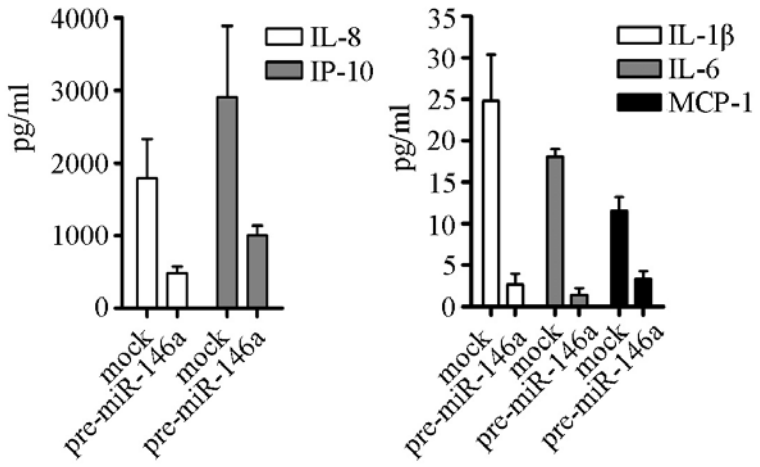

Figure 3-6. Pre-transfection of miR-146a in THP-1 cells reduces LPS-induced cytokine/chemokine production. THP-1 cells were transfected with pre-miR-146a, incubated for 48 hours, and then treated with LPS for 24 hours. Culture supernatants were collected from LPS treated and untreated cells. Multiplex analysis was used to quantitatively detect cytokines and chemokines as described in Methods. The cytokine/chemokine concentrations in LPS-treated culture supernatants were normalized to untreated culture supernatants and are shown in two scales. Mock represents level of cytokines/chemokine induced by LPS alone after 24 hours. THP-1 cells transfected with pre-miR-146a exhibited decreased production of cytokines. Average plus SD is shown, n=2 independent experiments.

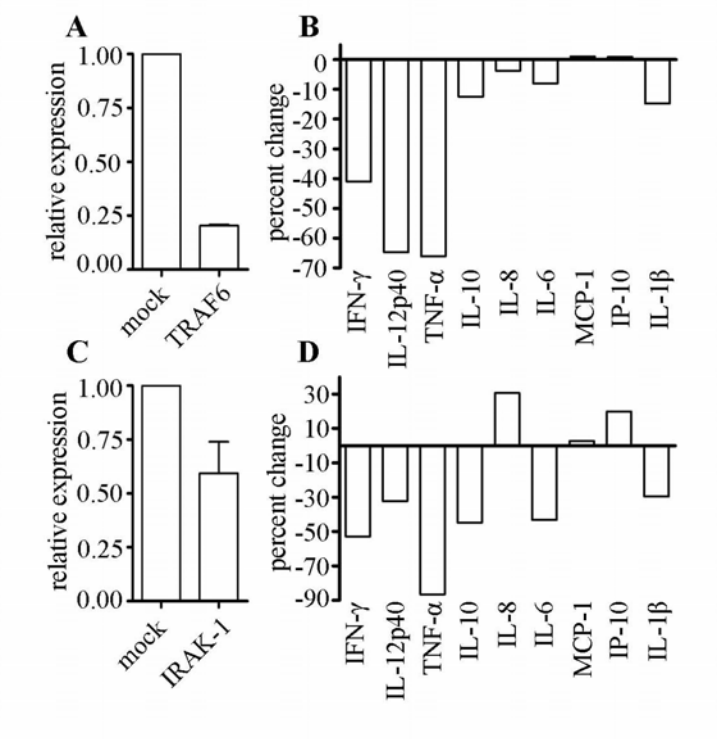

Figure 3-7. Knockdown of TRAF6 or IRAK-1 results in decreased production of certain cytokines. A and C) siRNA targeting TRAF6 or IRAK-1 was transfected into THP-1 cells, and knockdown efficiency was determined using qRT-PCR. B and D) 48 hours after transfection, mock and TRAF6 or IRAK-1 deficient cells were treated with LPS for 24 hours. Cytokine production was measured using a multiplex assay. Percent change between mock and TRAF6 deficient cells is shown.

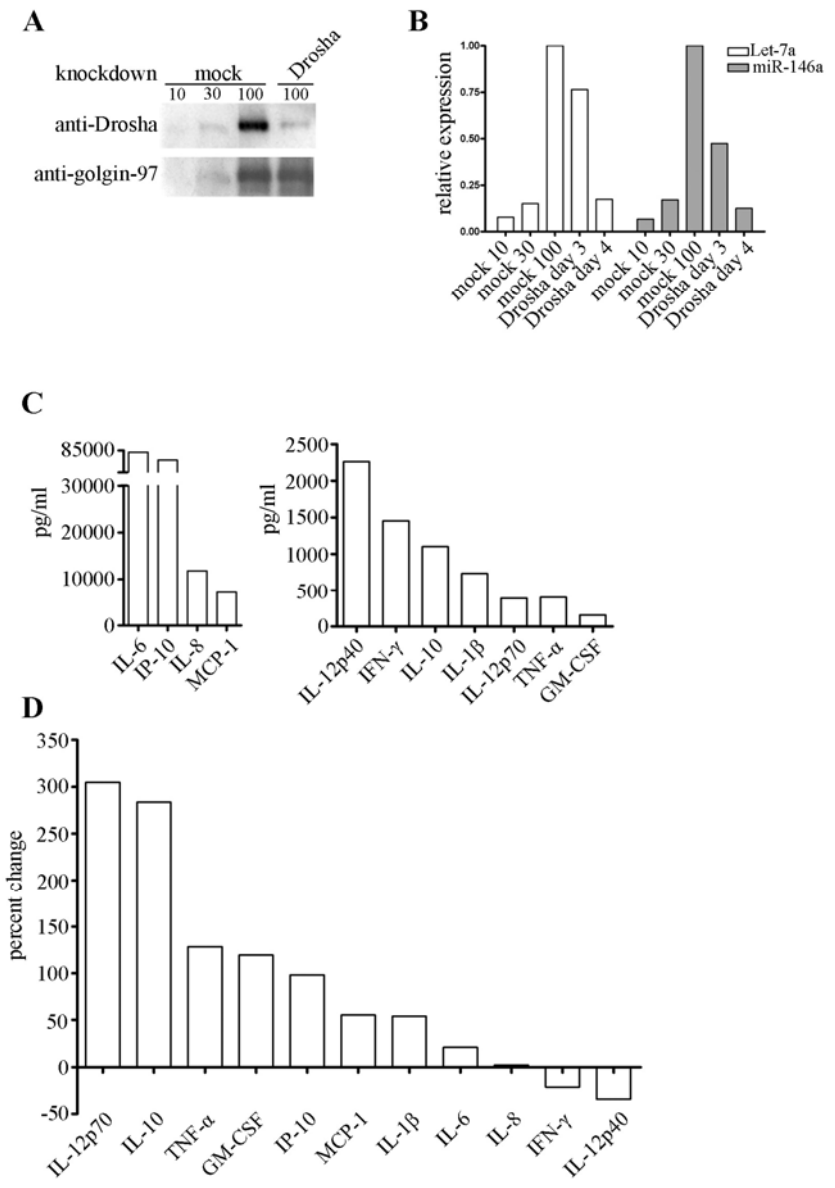

Figure 3-8. Depletion of miRNA results in increased LPS-induced cytokine production. Reduction of endogenous miRNA was achieved in THP-1 cells by siRNA knockdown of Drosha. (A) Reduction in level of Drosha protein shown by Western blot analysis comparing mock transfected cells with cells transfected with siRNA targeting Drosha. Golgin-97 levels are shown as loading control. (B) Comparison of miRNA levels in day 3 and 4 show Drosha knockdown resulted in depletion of miRNA Let-7a and miR-146a after 4 days as determined by qRT-PCR described in Methods. All subsequent assays were performed 4 days after siRNA transfection. Mock 10, 30, and 100 indicate percent of RNA input from mock transfected cells. (C) Cytokine and chemokine production in LPS-treated control THP-1 cell culture supernatant after 48 hours. Control cells were cultured in the presence or absence of LPS for 48 hours and their culture supernatants were harvested. Multiplex analysis was used to detect cytokines/chemokines as described in Methods. The

cytokine/chemokine concentrations in LPS-treated culture supernatants were normalized to untreated culture supernatants and are shown in two scales. (D) The percent change in cytokine/chemokine production was calculated by comparing culture supernatants of miRNA-depleted and mock transfected control cells.

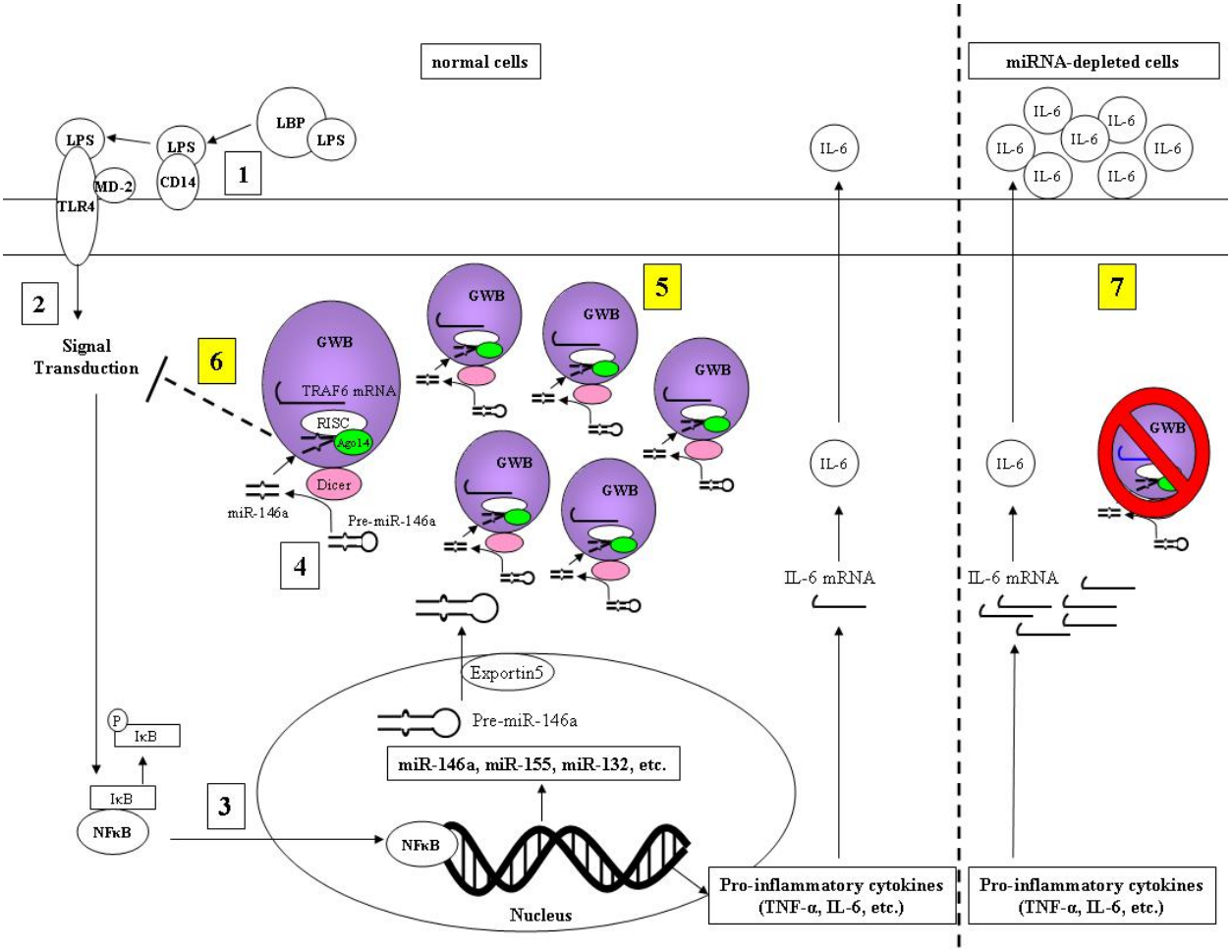

Figure 3-9. Model of LPS-induced GWB assembly. See text in Discussion.

CHAPTER 4
UPREGULATED MIR-146A EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM RHEUMATOID ARTHRITIS PATIENTS

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation of synovial tissue which results in irreversible joint damage (132). Inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), play an important role in RA pathogenesis, and inhibition of these cytokines can ameliorate disease in some patients (133,134).

MicroRNA (miRNA) are small non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional level (135,136). It is predicted that as much as one-third of all messenger RNAs (mRNAs) are targeted for miRNA-mediated regulation (3), and the importance of miRNA regulation is becoming increasingly clear as new roles in critical cellular processes such as apoptosis, differentiation, and the cell cycle are discovered.

The biogenesis and maturation of miRNA are dependent on two RNase III enzymes, Drosha and Dicer. First, miRNA are transcribed by RNA polymerase II into a long primary miRNA (pri-miRNA) transcript (10,11). The pri-miRNA is then cleaved by Drosha and its partner protein DGCR8 into a ~70 nucleotide (nt) precursor miRNA (pre-miRNA) molecule (12-16). The pre-miRNA is then exported into the cytoplasm via Exportin 5 where it is cleaved into a ~21 nucleotide miRNA duplex similar in structure to small interfering RNA (siRNA) (20,21). One strand of the miRNA duplex is then loaded into the RNA-induced silencing complex (RISC) where it binds the 3' untranslated region (3'UTR) of its target mRNA causing the degradation or translational repression of that mRNA (21).

The key components of RISC are the argonaute proteins 1-4 (Ago1-4). Ago2 is known to be the catalytic enzyme of RNA interference (RNAi) and is critical for both miRNA and siRNA

function (25,26). In addition to Ago2, many other proteins are critical for miRNA function, including GW182 and Rck/p54. These proteins, as well as miRNA and siRNA, localize to cytoplasmic foci known as GW or P bodies (here referred to as GWB). Our recent studies have established GWB to be useful biomarkers for siRNA and miRNA activity in cells (36,39). Our latest study demonstrated that the number and size of GWB significantly increases concurrently with increased miRNA expression in lipopolysaccharide (LPS)-treated THP-1 cells, implicating that GWB can be monitored as biomarkers for miRNA activity (Pauley et al. in preparation).

TNF-α stimulation has been shown to induce the expression of certain miRNA including miR-146a and miR-155, in monocytes and macrophages (40,42). Based on these data and the fact that TNF-α plays an important role in RA pathogenesis as supported by the development of successful anti-TNF-α therapies, we set out to examine miRNA expression in RA patients compared to healthy controls.

In this study, we obtained peripheral blood mononuclear cells (PBMCs) from RA patients and controls and examined the expression of miR-146a, miR-155, miR-132, miR-16, and miRNA let-7a. These miRNA were chosen for examination based on previous reports linking them to immune stimulation by LPS or TNF-α, or in the case of miR-16, its ability to target the 3'UTR of TNF-α (40,42,118). MiRNA let-7a was chosen as a control. This study is significant because it demonstrates that miRNA expression in RA PBMCs may mimic the conditions in synovial tissue, and thus enable us to bypass the need for synovial tissue samples allowing for the analysis of larger patient populations.

Material and Methods

Patients and Controls

Sixteen patients who fulfilled the American College of Rheumatology classification criteria for RA were included in the study. Their demographic and clinical information is

included in Table 4-1. Four disease controls, including one SLE, two Sjögren's syndrome (SjS), and one systemic sclerosis (SSc), were included. Nine healthy donors with no history of autoimmune disease were included as controls. This study was approved by the University of Florida Institutional Review Board, and written permission was obtained from all subjects who participated in the study.

Collection of PBMC and qRT-PCR

Blood samples were collected in EDTA-treated tubes and PBMCs were isolated by standard Ficoll density-gradient centrifugation. PBMCs were washed once in sterile PBS before culture or RNA isolation. Total RNA was isolated from freshly obtained PBMCs using the *mir*Vana miRNA Isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentrations were determined and equal amounts of each RNA sample were used for qRT-PCR. MiRNA qRT-PCR was performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix, and TaqMan MicroRNA Assay primers for human miR-146a, miR-155, miR-132, miR-16 and let-7a (Applied Biosystems, Foster City, CA). mRNA qRT-PCR was performed using the TaqMan High-Capacity cDNA Reverse Transcription Kit, TaqMan Fast PCR Master Mix, and TaqMan mRNA assay primers (Applied Biosystems). All reactions were analyzed using StepOne Real-Time PCR System (Applied Biosystems). The levels of miRNA were normalized to U44 controls whereas mRNA levels were normalized to 18S RNA. The cycle threshold (Ct) values, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission, were determined and the relative miRNA or mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method (126).

Cell Culture and Cytokine Treatment

THP-1 human monocytes obtained from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum. THP-1 cells were seeded at $5x10^5$ cells per well in a six-well plate and treated with 10 ng/ml of TNF-α, IFN-γ, IL-12p70, IL-4, IL-10 (BD Biosciences, San Jose, CA), IFN-α, IFN-β (PBL Interferon Source, New Brunswick, NJ), or M-CSF (US Biological, Swampscott, MA). Cells were also treated with 25 ng/ml MCP-1 (Sigma) in serum free media. After designated treatment time, cells were harvested and washed once in PBS prior to analysis.

Indirect Immunofluorescence (IIF)

The THP-1 cells were cytospun onto glass slides at 1,000 rpm for 5 minutes. PBMCs were cultured on glass slides at 37°C for 1 hour. Cells were fixed in 3% paraformaldehyde for 10 minutes and permeabilized in 0.5% Triton X-100 for 5 minutes. GWB were detected in THP-1 cells with a human prototype anti-GWB serum (28) used at 1:6000 dilution, and in PBMCs with rabbit anti-Rck/p54 antibodies used at 1:500 dilution. TRAF6 and IRAK-1 were detected using rabbit anti-TRAF6 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-IRAK-1 (1:50, Santa Cruz Biotechnology). Secondary antibodies used were Alexa Fluor 488 goat anti-human IgG or goat anti-rabbit IgG (1:400) from Molecular Probes (Carlsbad, CA). Slides were mounted using Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (VECTOR Laboratories, Burlingame, CA). Fluorescence images were taken with Zeiss Axiovert 200M microscope and a Zeiss AxioCam MRm camera using the 20x or 40x 0.75 NA objectives. Color images were assessed using Adobe Photoshop version 7. GWB were counted using Cell-Profiler image analysis software (123).

Transfection of siRNA

The siRNAs targeting TRAF6 and IRAK-1 were transfected into THP-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To monitor the transfection efficiency, Cy3-labeled siRNA targeting lamin A/C was transfected into cells in parallel in all transfections, and at least 80% transfection efficiency was achieved. The siRNAs used in this study were all purchased from Applied Biosystems. The sense and antisense strand sequences are listed below: IRAK-1: 5'-GGUUUCGUCACCCAAACAUtt-3' and 5'AUGUUUGGGUGACGAAACCtg-3'; TRAF6: 5'-GGUUGUUUGCACAAGAUGGtt-3' and 5'-CCAUCUUGUGCAAACAACCtt-3'.

Multiplex Analysis of Cytokines

The THP-1 cells were transfected as described above, and then treated with 1 μg/ml LPS (*Salmonella enterica* serotype minnesota, Sigma, St. Louis, MO) for 24 hours in culture medium. The culture supernatant was then harvested and frozen at -80°C for storage before multiplex analysis. The human cytokine/chemokine LINCOplex premixed kit (LINCO Research, St. Charles, MO) was used according to the manufacturer's protocol to quantitatively detect human MCP-1and TNF-α.

Results and Discussion

Specific Cytokines/Chemokines Induce GWB in THP-1 Cells and Human PBMCs

Since our previous work demonstrating that GWB can be used as biomarkers for miRNA activity (Pauley et al. in preparation), we began examining a variety of cytokines and chemokines for their ability to stimulate miRNA activity in human monocytic THP-1 cells. THP-1 cells were treated with 10 ng/ml TNF-α, IFN-α, IFN-β, IFN-γ, IL-12p70, M-CSF, IL-4, and IL-10 or 25 ng/ml MCP-1 for four hours. IIF was performed using a human anti-GWB serum to detect GWB in the cells. As shown in Figure 4-1A, the pro-inflammatory

cytokines/chemokines TNF-α, IFN-α, IFN-β, IFN-γ, and MCP-1 resulted in a significant increase in the number of GWB per cell compared to untreated cells cultured in parallel ($P<0.0001$ as determined by one-way ANOVA). However, IL-12p70, M-CSF, IL-4 and IL-10 had no significant effect on the number of GWB. TNF-α elicited the strongest response in THP-1 cells with 4-fold increase in the average number of GWB per cell (Figure 4-1A and C). These experiments were repeated at least three times with reproducible results each time.

Next, we decided to examine the effect of TNF-α stimulation on human PBMC GWB. GWB staining using human PBMCs from a healthy donor, after 4 hours stimulation with TNF-α (1 ng/ml) is shown. Similar to THP-1 cells, the number of GWB per cell increased 3.5 fold after TNF-α stimulation of PBMCs (Figure 4-1B and C, $P<0.0001$ as determined by Mann Whitney test). This data indicates that THP-1 cells may be suitable to substitute for human PBMCs in some of the subsequent experiments.

Increased Expression of miR-146a, miR-155, miR-132, and miR-16 in RA Patient PBMCs

In Figure 4-1 we have shown that TNF-α is a potent inducer of GWB and therefore, miRNA activity. Our preliminary studies and work from other investigators have confirmed that TNF-α stimulation induces the expression of certain miRNA including miR-146a and miR-155 (40,42). Based on these data and the important role of TNF-α in RA pathogenesis and therapies, we began to investigate the expression levels of miRNA in RA patients compared to healthy and disease controls. PBMCs were obtained from patients (n=17 RA, n=4 disease controls) and healthy donors (n=9) and isolated by Ficoll density-gradient centrifugation. Initially, RA PBMCs were monitored by IIF for GWB; however, we did not observe an increased number of GWB in RA compared to healthy controls (not shown). This discrepancy could be due to the limited sensitivity in the quantitation of GWB.

As shown in Figure 4-2A, the average relative expression of miR-146a, miR-155, miR-132 and miR-16 was 2.6, 1.8, 2.0, and 1.9 fold, respectively, higher for RA patients compared to healthy controls ($P<0.01$ for miR-146a, $P<0.05$ for miR-155, miR-132, and miR-16 as determined by one-way ANOVA). The expression of miRNA let-7a was not significantly different between RA patients and healthy controls (Figure 4-2A). Disease control miRNA expression resembled that of healthy controls.

To examine the relationship between RA disease activity and miRNA expression levels, patients were classified into inactive/remission and active patients based on C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) values. Three patients with normal CRP and ESR (Table 4-1) were classified as inactive, while eight patients with elevated CRP and/or ESR (Table 4-1) were classified as active (Figure 4-2B). Those patients with incomplete or no available data were omitted (Table 4-1). MiRNA expression levels were compared between the groups. Interestingly, high miR-146a and miR-16 expression seemed to correlate with active disease, while low expression correlated with inactive disease (Figure 4-2B, $p<0.05$ as determined by t-test). Although larger patient populations are needed for this analysis, this data indicates that miR-146a and miR-16 expression levels may be a useful marker of RA disease activity.

Figure 4-2C shows miRNA expression of two samples from a single RA patient collected over a two month interval. The miRNA levels of this patient were largely unchanged over the two month interval, indicating that miRNA expression in RA patients may be consistent over time. In this patient, miR-146a, miR-155, and miR-132 expression was stable over this time period, while the expression of miR-16 and miRNA let-7a increased approximately 3.5 and 2.4-

fold, respectively. However, a larger patient population must be examined in order to validate this data.

To further analyze the increased miRNA expression in these RA patients, we compared miR-146a, miR-155, and miR-132 expression levels with patient clinical and demographic information (Table 4-1) and found no significant trends or correlations between high expression levels and age, race, or medications. Patients receiving no medications at the time of miRNA analysis exhibited the same trend of elevated miRNA expression indicating that treatment with medications is not responsible for the increased miRNA expression in RA patients.

Recently, two reports have shown increased miR-146 and miR-155 expression in RA synovial tissue and fibroblasts (137,138). Stanczyk et al. reported a 4-fold increase in miR-146a expression and a 2-fold increase in miR-155 expression in RA synovial fibroblasts compared to osteoarthritis synovial fibroblasts, and demonstrated that miR-155 expression can repress the induction of matrix metalloproteinases 3 and 1 indicating that miR-155 may be involved in modulating the destructive properties of RA synovial fibroblasts (138). However, in this report, miR-155 expression from RA PBMCs was not significantly different from control PBMCs (138). This discrepancy could be due to differences in experimental techniques or patient populations. Nakasa et al. also reported an approximately 4-fold increase in miR-146a expression in RA synovial tissue (137). Our data demonstrate that RA patient PBMCs exhibit elevated miRNA expression in a similar manner to RA synovial tissue with a 2.6-fold increase in miR-146a expression and a 1.8-fold increase in miR-155 expression. Due to the invasiveness involved in collecting samples, monitoring miRNA expression in RA synovial tissue is, in most cases, limited to extremely severe patients undergoing joint surgery or replacement. Since blood

collection is not invasive this allows for easy sample collection over time which is a distinct advantage when monitoring disease activity and treatment efficacy.

Monocyte/Macrophage Population of RA PBMCs Exhibits Increased miRNA Expression

Since PBMCs are composed of a mixed cell population, the two main components of which are monocytes/macrophages and lymphocytes, we wanted to determine which cell population in RA patients exhibits increased miRNA expression. PBMCs were isolated from RA patients (n=2) and incubated in tissue culture dishes at 37°C for 1 hour. The monocyte/macrophage population adhered to the dish, while lymphocytes remained in suspension. The adherent cells were washed five times with sterile PBS, and the non-adherent cells were collected and washed with sterile PBS. The purity of the adherent population was approximately 80% as determined by microscopy. RNA was isolated from the cells, miRNA expression was analyzed by qRT-PCR, and the data was normalized within the total group of patient and control samples. The expression of miR-146a, miR-155, miR-132, and miR-16 was 2.8, 1.6, 4.2, and 3.4-fold higher, respectively, in monocytes compared to lymphocytes (Figure 4-3, $P<0.02$ as determined by Mann-Whitney test). Let-7a expression was similar between monocytes and lymphocytes (not shown). Figure 4-3B shows the average expression (miR-146a, miR-155, miR-132, and miR-16 combined) for the monocyte and lymphocyte populations of two RA patients ($P<0.02$ as determined by Mann-Whitney test). These results suggest that monocytes/macrophages contribute to the increased miRNA expression observed in RA patients more than lymphocytes, but further studies need to be performed to confirm this observation.

Expression of TRAF6 and IRAK-1 Is Similar Between RA Patients and Controls

Since the majority of the RA patients exhibited increased expression of miR-146a compared to healthy and disease controls, we decided to examine the expression of two targets of

miR-146a, TRAF6 and IRAK-1. TRAF6 and IRAK-1 mRNA expression was analyzed by qRT-PCR (Figure 4-4A and B). Since RA patients exhibited increased miR-146a production compared to controls, we expected to observe decreased TRAF6 and/or IRAK-1 expression in RA patients compared to controls, and overall the mRNA levels of TRAF6 and IRAK-1 do not exhibit the same degree of variability between patients that we observed with miRNA expression. This may indicate that TRAF6 and IRAK-1 transcripts are under other levels of control. However, TRAF6 and IRAK-1 mRNA expression was very similar between RA patients and controls. To confirm this discrepancy, we analyzed TRAF6 and IRAK-1 protein levels by IIF in one healthy control and one RA patient whose miR-146a level was increased (Figure 4-2A). PBMCs were processed for IIF as previously described and were stained for TRAF6 and IRAK-1. Image J software was used to quantify the relative level of fluorescence for at least 20 cells. As shown in Figure 4-4C, there was no significant difference in TRAF6 or IRAK-1 protein levels between the RA patient and healthy control, which is consistent with the mRNA analysis.

It is interesting to speculate that this lack of regulation of TRAF6/IRAK-1 by miR-146a could play a role in RA pathogenesis, especially since it has been reported that inhibition of IRAK-1 using antisense oligonucleotides results in decreased LPS-induced cytokine production (139) and our previous studies have shown that transfection of miR-146a into THP-1 monocytes results in knockdown of TRAF6 and IRAK-1 expression and inflammatory cytokine production. To further investigate this possibility, we transfected siRNA targeting TRAF6 and/or IRAK-1 into THP-1 cells. The knockdown efficiency was determined by analyzing TRAF6 and IRAK-1 mRNA levels by qRT-PCR and at least 80% and 60% knockdown was achieved for TRAF6 and IRAK-1, respectively (Figure 4-5A). Two days after transfection, knockdown and control cells

were treated with 1 μg/ml LPS for 24 hours. Culture supernatants were collected and cytokines/chemokines were quantitatively detected using a human cytokine multiplex assay. TNF-α production was drastically reduced in the TRAF6 and/or IRAK-1 deficient cells compared to mock transfected cells (Figure 4-5B), while MCP-1 production was not affected by TRAF6 or IRAK-1 knockdown (Figure 4-5C). This data demonstrates that TRAF6 and IRAK-1 are required for the production of TNF-α in THP-1 cells. Taken together, it is reasonable to hypothesize that the absence of TRAF6/IRAK-1 regulation by miR-146a in RA patients could contribute to the prolonged production of TNF-α that many of these patients exhibit. Furthermore, it would be interesting to investigate the expression patterns of miR-146a, TRAF6 and IRAK-1 in RA patients who are responsive to anti-TNF-α therapy versus those who are not responsive. Clearly, further studies are needed to elucidate the role of miR-146a regulation in RA pathogenesis and the mechanism by which TRAF6/IRAK-1 escape miR-146a regulation.

Conclusions

In summary, this study has shown that RA patient PBMCs exhibit statistically significant increased expression of miR-146a, miR-155, miR-132 and miR-16 compared to healthy and disease controls. Furthermore, we demonstrate that although miR-146a expression is increased in RA patients, levels of the two established miR-146a targets TRAF6 and IRAK-1 in RA patients are similar to controls. We also show that TRAF6 and IRAK-1 regulation is important for TNF-α production in THP-1 cells.

Normally, stimulus such as LPS or TNF-α will induce the expression of miR-146a, miR-155, and miR-132 in a NFκB-dependent manner (40). In the case of miR-146a, this will lead to the negative regulation of TRAF6 and IRAK-1, which in turn will decrease the production of pro-inflammatory cytokines/chemokines including TNF-α. Thus the function of miR-146a, at least in part, is to control the extent of the stimulation such that the production of some of these

pro-inflammatory cytokines/chemokines will not continue for an extended period of time. However, in RA patients, the increased miR-146a is unable to negatively regulate TRAF6 or IRAK-1 which may lead to prolonged TNF-α production and disease progression.

While it is very exciting that two independent studies have shown increased miRNA expression in RA synovial tissue, analyzing miRNA expression in patient PBMCs presents a distinct advantage over analyzing synovial tissue samples. Collection of PBMCs is non-invasive, and samples can be collected from patients ranging in disease severity from early onset to more severe, while synovial tissue collection is biased towards patients with severe degenerative disease. With further validations and studies on larger patient populations, monitoring of selected miRNA could prove to be a valuable addition to RA diagnostics, or monitoring disease progression or treatment efficacy. The underlying mechanisms resulting in increased miRNA expression and inability of miR-146a to regulate its targets need to be elucidated, and these mechanisms may be potential targets for the development of new RA therapies.

This work has been submitted for publication in 2008 in *Arthritis Research and Therapy*.

Table 4-1. Demographic and clinical information of patients*

Subject	*Sex*	*Age*	*Medications*	*CRP*	*ESR*
RA Patients					
RA-1a[1]	M	65	None	4.14[2]	9
RA-1b	M	65	MTX	11.8	18
RA-2	F	33	Enbrel; Naproxen	25.7	18
RA-3	F	43	Enbrel	No data	No data
RA-4	F	45	None	98.4	84
RA-5	F	55	MTX	4.8	26
RA-6	F	73	None	2.1	30
RA-7	F	51	Plaquenil; MTX; Prednisone	No data	15
RA-8	M	61	Prednisone; MTX; Plaquenil; Azalfadine	No data	1
RA-9	M	46	Medrol; MTX; Enbrel	2	9
RA-10	F	32	Arava	94.2	63
RA-11	F	50	Enbrel; MTX	No data	No data
RA-12	M	67	MTX; Celebrex; Prednisone	0.6	11
RA-13	F	55	MTX	No data	17
RA-14	F	33	Plaquenil; Motrin	2.8	7
RA-15	F	55	None	168.2	88
RA-16	F	56	Prednisone; Minocycline	No data	No data
Disease controls					
SLE1	F	36	MTX; Plaquenil; Redinizine		
SjS1	F	30	Plaquenil; Cellcept		
SjS2	M	21	Plaquenil		
SSc1	F	57	Cyclosporine; Plaquenil; Prednisone; Cellcept		

*CRP= C-reactive protein; ESR= erythrocyte sedimentation rate; RA= rheumatoid arthritis; SLE= systemic lupus erythematosus; SjS= Sjögren's syndrome; SSc= scleroderma; MTX= methotrexate. [1]Sample collected from RA-1 before and after MTX treatment. [2]Normal value less than 0.8. Normal CRP<4.9 mg/L, normal ESR<20 mm/H for females, <10 mm/H for males.

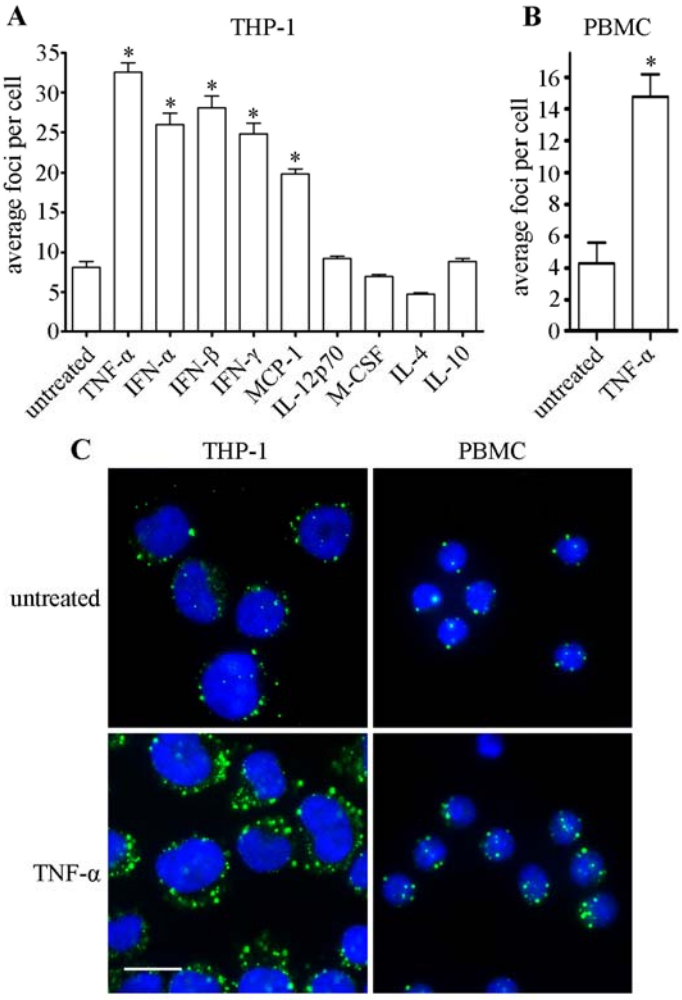

Figure 4-1. Stimulation with TNF-α results in increased number of GWB in THP-1 and human PBMCs. A) THP-1 cells were treated with 10 ng/ml TNF-α, IFN-α, IFN-β, IFN-γ, IL-12p70, M-CSF, IL-4, IL-10 or 25 ng/ml MCP-1 for four hours. IIF was performed using a human anti-GWB serum to detect GWB, and the number of GWB were counted using CellProfiler image analysis software. Average number of GWB per cell and SEM is shown. Asterisks (*) indicate $P<0.0001$ as determined by one-way ANOVA. B) Human PBMCs were obtained from a healthy donor and isolated using Ficoll density-gradient centrifugation. The cells were then cultured four hours in the presence of 1 ng/ml TNF-α. GWB were detected by IIF using rabbit anti-Rck/p54 antibodies. Average number of GWB and SEM is shown. Asterisk (*) indicates $P<0.0001$ as determined by Mann-Whitney test. C) IIF image of THP-1 and PBMCs treated with 10 ng/ml or 1 ng/ml TNF-α for four hours, respectively. GWB shown in green, nuclei counterstained with DAPI (blue). Bar, 10 μm.

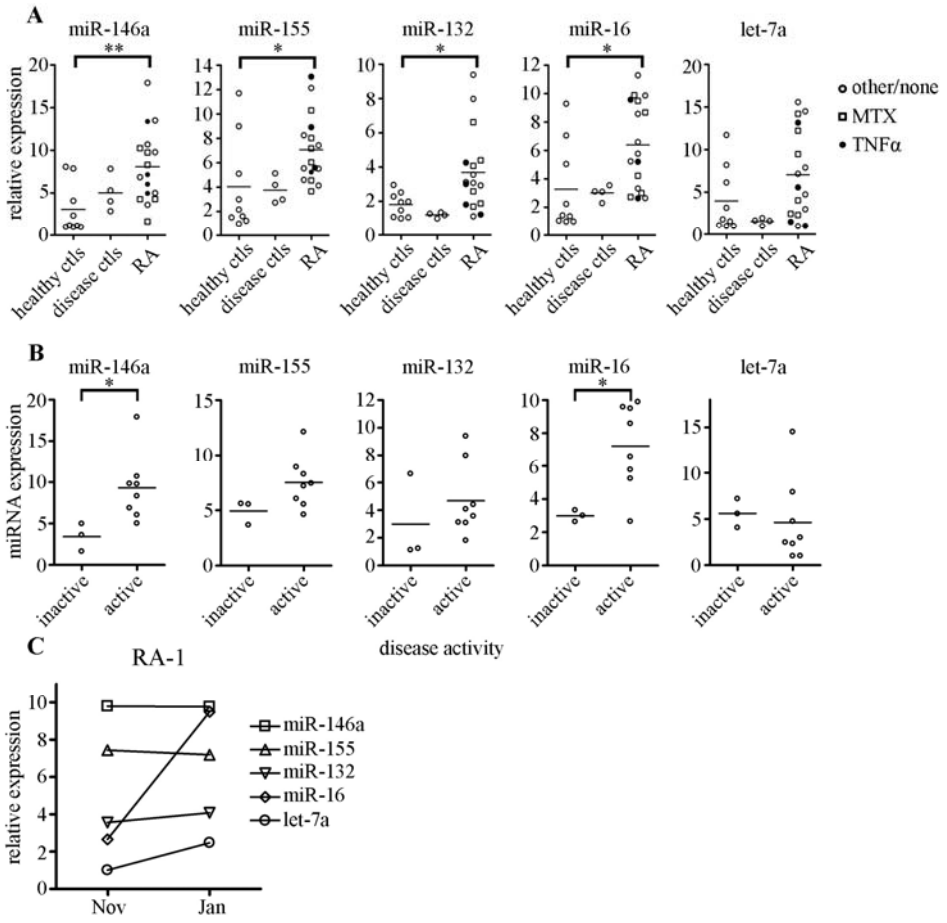

Figure 4-2. Aberrant expression of miR-146a, miR-155, miR-132 and miR-16 is exhibited by RA patient PBMCs compared to healthy controls. A) RNA was isolated from healthy control (n=9), disease control (n=4) and RA patient (n=17) PBMCs, and relative expression levels of miR-146a, miR-155, miR-132, miR-16, and miRNA let-7a were analyzed by qRT-PCR using U44 RNA as an internal control. Average is indicated by bars. Asterisks (*) indicate $P<0.05$, (**) indicates $P<0.01$ as determined by one-way ANOVA. For RA patients, closed circles indicate patients undergoing anti-TNF-α therapy at time of sample collection, squares indicate methotrexate (MTX) treatment, and open circles indicate other or no treatment. B) Disease activity was determined for patients using CRP and ESR values and correlated with miRNA expression. Normal CRP and ESR values were classified as inactive disease (n=3, Table 1, patients 9, 12, and 14), and higher than normal CRP or ESR values were classified as active disease (n=8, Table 1, patients 1a, 1b, 2, 4, 5, 6, 10, and 15). Those patients with no or incomplete data for CRP/ESR values were omitted. Asterisk (*) indicates $p<0.05$ as determined by t-test. C) PBMCs were collected from patient RA-1 before (November, 2007) and after (January, 2008) MTX treatment and miRNA expression was examined using qRT-PCR. miRNA expression is largely consistent over time with the exception of increased miR-16 expression.

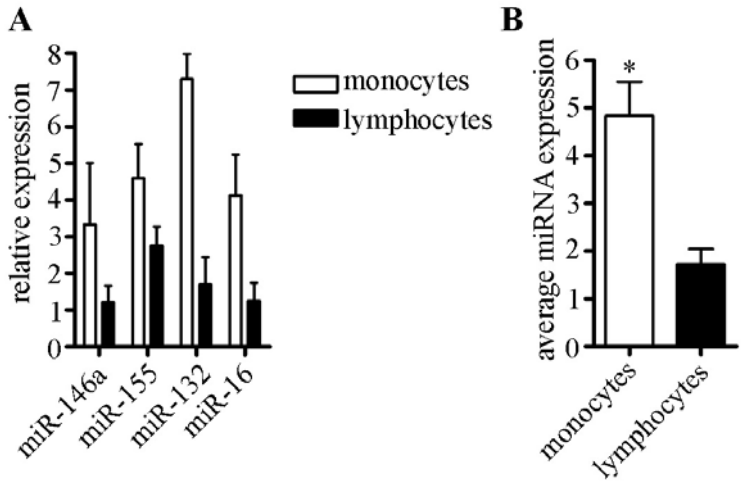

Figure 4-3. Monocyte/macrophage fraction of PBMCs exhibit increased miRNA expression compared to lymphocyte fraction. PBMCs were collected from RA patients and separated into monocyte/macrophage and lymphocyte populations by allowing the monocytes/macrophages to adhere to a tissue culture dish. A) miRNA expression was examined using qRT-PCR. SEM is shown, n=2 patients. B) Average expression of miR-146a, miR-155, miR-132 and miR-16 is shown for monocyte and lymphocyte populations for 2 RA patients. Asterisk (*) indicate $P<0.02$ as determined by Mann-Whitney test. SEM is shown.

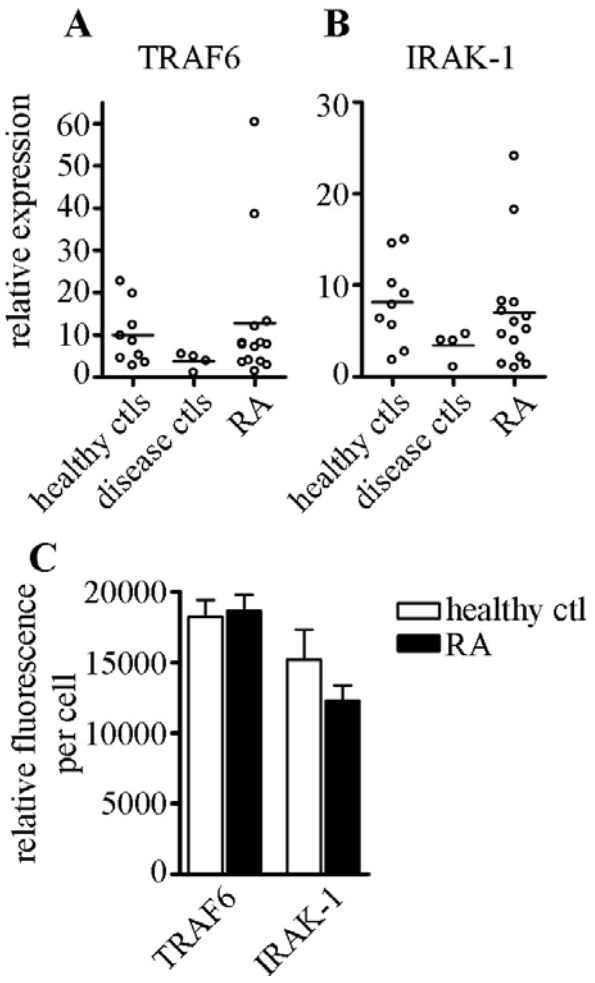

Figure 4-4. Expression levels of TRAF6 and IRAK-1 are similar between RA patients, healthy controls and disease controls. RNA was isolated from healthy control (n=9), disease control (n=4) and RA patient (n=14) PBMCs and mRNA expression levels of TRAF6 (A) and IRAK-1 (B) were analyzed using qRT-PCR. C) PBMCs isolated from a healthy control and RA patient were incubated on glass slides for 1 hour at 37°C. The adhered cells were fixed and permeabilized in 3% paraformaldehyde and 0.5% Triton X-100, respectively. Protein levels of TRAF6 and IRAK-1 were analyzed by immunofluorescence using rabbit anti-TRAF6 and anti-IRAK-1 antibodies, and relative fluorescence was determined using ImageJ analysis software. SEM is shown, n> 20 cells.

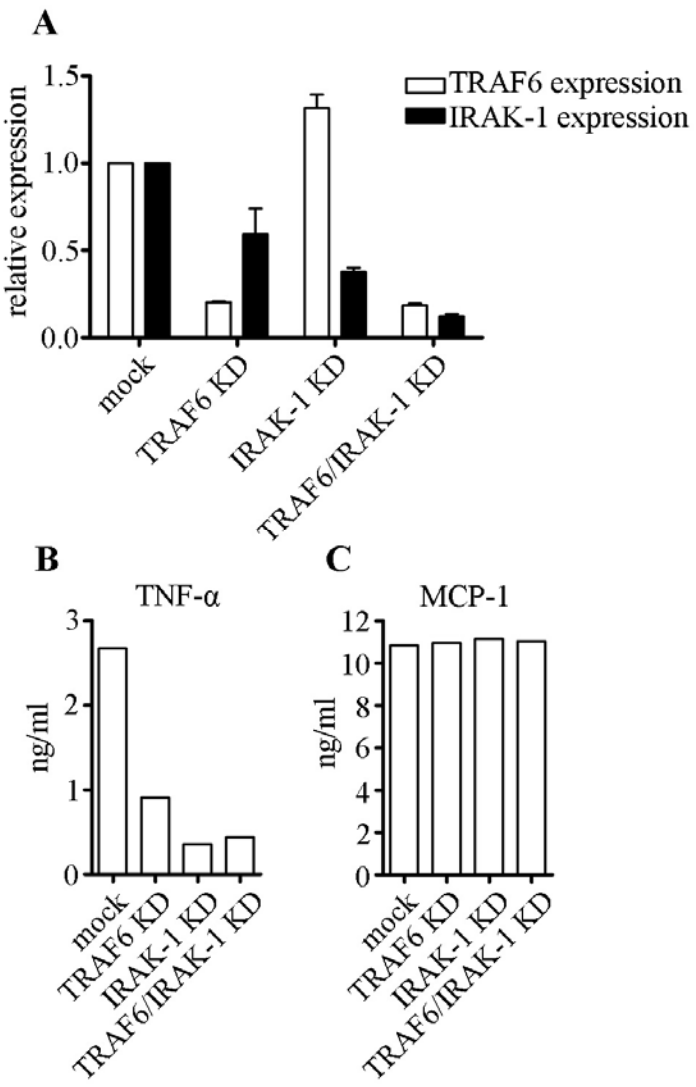

Figure 4-5. Knockdown of TRAF6 and/or IRAK-1 results in decreased TNF-α production in THP-1 cells. THP-1 cells were transfected with siRNA targeting TRAF6 and/or IRAK-1. A) 48 hours after transfection, mRNA levels of TRAF6 and IRAK-1 were analyzed by qRT-PCR and normalized to mock transfected cells. SEM shown, n=2. After knockdown of TRAF6 and/or IRAK-1 was confirmed by qRT-PCR, cells were treated with 1 μg/ml LPS for 24 hours and culture supernatants were collected. Multiplex assay was used to quantitatively detect TNF-α (B) and MCP-1 (C).

CHAPTER 5
CONCLUSIONS

Formation of GWB is a Consequence of miRNA Genesis

This study first examined the relationship between GWB and miRNA. Previous to our study, GWB were believed to be involved in mRNA storage and/or degradation and were only linked to RNAi by the localization of RNAi and miRNA-related proteins such as GW182 and Ago2. Our study set out to further characterize the role of GWB in the endogenous miRNA pathway. We found that endogenous let-7 miRNA co-precipitated with the GW182 protein complex, and that miRNA transfected into cells localizes to GWB (36). Depletion of endogenous miRNA was achieved by knocking down Drosha or its partner protein DGCR8, both of which are critical for the generation of mature miRNA, and resulted in the loss of GWB formation (36). However, subsequent introduction of siRNA targeting lamin A/C as a surrogate for miRNA resulted in the reappearance of GWB and the concurrent knockdown of lamin A/C protein (36). These data indicate that GWB formation is dependent on the presence of small RNA (siRNA or miRNA) in the cells.

This work has significantly contributed to the understanding of the role of GWB in miRNA function within the cell. This study was performed in a mammalian cell system and published in *EMBO Reports* in 2006 (36), and recently, another group has reproduced our results in Drosophila cells. Eulalio et al. depleted miRNA from Drosophila S2 cells by using siRNA to knock down Drosha or Pasha, the Drosophila ortholog of DGCR8, and found that GWB were disassembled (116). They go on to propose that in Drosophila, GWB are a consequence of miRNA and siRNA activity, therefore disrupting the siRNA or miRNA pathway at any step prevents the formation of GWB (116). Notably, this group uses siRNA to knockdown Drosha and Pasha compared to our technique of using shRNA to knockdown these genes.

In the mammalian system, further studies need to be performed in order to determine whether or not GWB are required for miRNA function, or if they form as a result of miRNA. However, sometimes *in vitro* manipulations do not yield an accurate depiction of *in vivo* cellular conditions. For example, in Drosophila, GWB are not required for miRNA/siRNA function as demonstrated by the functionality of RNA-mediated gene silencing in cells lacking detectable microscopic GWB (116). *In vivo*, however, GWB form in order to provide a microenvironment for this process. One can argue that despite the lack of requirement for GWB in miRNA/siRNA function under artificial *in vitro* conditions, the important fact of the matter is that *in vivo*, GWB formation does occur in response to miRNA/siRNA activity, perhaps in order to allow for these processes to occur more efficiently by localizing all of the proteins involved to one cytoplasmic foci rather than having them scattered throughout the cytoplasm.

Formation of GW/P Bodies as Marker for miRNA-Mediated Regulation of Innate Immune Signaling

The next part of this study examined the effects of LPS stimulation of human monocytes on GWB formation, miRNA induction, miRNA target regulation, and downstream cytokine and chemokine expression. We found that the number and size of GWB significantly increased in response to LPS stimulation and this increase was abolished when the miRNA-effector proteins Rck/p54 or Ago2 were depleted (140). When miRNA was depleted in THP-1 cells by knocking down Drosha, cytokine production was significantly altered (140). Since the level of miR-146a increased by 100 fold during LPS stimulation, we examined the transfection of precursor miR-146a alone and showed similar increases in GWB and reduction in major cytokines/chemokines induced by LPS, implicating that miR-146a alone could be responsible for this effect (140). These data show that the size and number of GWB serve as a biomarker for miRNA mediated

gene regulation, and miRNA play a significant role in the regulation of LPS-induced cytokine production in human monocytes.

The ability to monitor GWB as markers for miRNA activity provides a useful technique for screening different treatments or cellular conditions for their effect on miRNA activity before performing costly and time consuming microarray studies. We have employed this technique to screen various cytokines and chemokines for their ability to induce miRNA activity (Figure 4-1A).

This study, which has been submitted for publication in *Molecular Biology of the Cell*, is the first to demonstrate that miRNA plays a role in regulating LPS-induced cytokine/chemokine production in monocytes. This finding is significant since the regulation of cytokine/chemokine production is critical in maintaining normal immune function. Overproduction of inflammatory cytokines/chemokines can result in cellular damage and apoptosis, and plays a role in the pathogenesis of many autoimmune diseases such as SLE and RA. Regulation of this process by miRNA permits for the fine-tuning of the inflammatory response.

Based on our data shown in Figure 3-3, that expression of miR-146a is increased up to 100-fold after 24 hours of LPS stimulation, it is interesting to speculate that miR-146a may also play a role in endotoxin tolerance. Endotoxin tolerance is a well known phenomenon in which repeated exposure to endotoxin results in a diminished immune response, usually characterized by a reduction in pro-inflammatory cytokine production (141). This phenomenon is thought to have developed as a protective evolutionary mechanism to prevent death caused by overwhelming cytokine release in sepsis (141). Interestingly, a recent report demonstrated that in PBMCs from young rats, IRAK-1 expression is decreased under endotoxin tolerance conditions (142). Since we have demonstrated that miR-146a levels are high up to 24 hours after

LPS stimulation, and we have shown that miR-146a can negatively regulate IRAK-1, it is possible that miR-146a regulation is important for endotoxin tolerance. However, further work is needed to determine if this hypothesis is correct.

Upregulation of miR-146a Expression in Peripheral Blood Mononuclear Cells From Rheumatoid Arthritis Patients

In the final part of this study, the expression of selected miRNA was examined in RA. Total RNA was isolated from PBMCs obtained from RA patients, healthy, and disease controls and the expression of miR-146a, miR-155, miR-132, miR-16, and miRNA let-7a was analyzed using qRT-PCR. RA PBMCs exhibited between 1.8 to 2.6 fold increase in miR-146a, miR-155, miR-132, and miR-16 expression, while let-7a expression was not significantly different compared to healthy controls. In addition, two targets of miR-146a, TRAF6 and IRAK-1, were similarly expressed between RA patients and controls, despite the increased expression of miR-146a in RA patients. Repression of TRAF6 and/or IRAK-1 in THP-1 cells resulted in up to an 86% reduction in TNF-α production, implicating that normal miR-146a function is critical for the regulation of TNF-α production. These data suggest a possible mechanism contributing to RA pathogenesis where miR-146a expression is increased but unable to properly function, leading to prolonged TNF-α production in RA patients. Recent studies have shown that RA patient synovial tissue and synovial fibroblasts exhibit increased expression of certain miRNA. Our data thus demonstrate that miRNA expression in RA PBMCs mimics that of synovial tissue/fibroblasts. The increased miRNA expression in RA patients is potentially useful as a marker for disease diagnosis, progression, or treatment efficacy.

This study, which has been submitted for publication in *Arthritis Research and Therapy*, is significant for several reasons. First, this is the first study to demonstrate differential miRNA expression in RA PBMCs. With further validations and studies on larger patient populations,

monitoring of selected miRNA could prove to be a valuable addition to RA diagnostics, or monitoring disease progression or treatment efficacy. Second, we have demonstrated a defect in miR-146a's ability to regulate TRAF6 and IRAK-1 in RA patients. This defective regulation could lead to prolonged and increased TNF-α production and disease progression in RA patients. Finally, we have shown that miRNA expression in RA PBMCs may mimic that of synovial tissue or fibroblasts. Analyzing miRNA expression in patient PBMCs presents a distinct advantage over analyzing synovial tissue samples since collection of PBMCs is non-invasive, and samples can be collected from patients ranging in disease severity from early onset to more severe, while synovial tissue collection is biased towards patients with severe degenerative disease.

REFERENCES

1. Griffiths-Jones, S. 2004. The microRNA Registry. *Nucleic. Acids. Res.* 32:D109-D111.

2. Griffiths-Jones, S., R. J. Grocock, S. van Dongen, A. Bateman, and A. J. Enright. 2006. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic. Acids. Res.* 34:D140-D144.

3. Lewis, B. P., C. B. Burge, and D. P. Bartel. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 120:15-20.

4. Kim, V. N., and J. W. Nam. 2006. Genomics of microRNA. *Trends. Genet.* 22:165-173.

5. Kim, Y. K., and V. N. Kim. 2007. Processing of intronic microRNAs. *EMBO. J.* 26:775-783.

6. Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst, and A. Bradley. 2004. Identification of mammalian microRNA host genes and transcription units. *Genome. Res.* 14:1902-1910.

7. Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell.* 75:843-854.

8. Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. 2000. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature.* 403:901-906.

9. Ambros, V., and R. C. Lee. 2004. Identification of microRNAs and other tiny noncoding RNAs by cDNA cloning. *Methods. Mol. Biol.* 265:131-158.

10. Cai, X., C. H. Hagedorn, and B. R. Cullen. 2004. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA.* 10:1957-1966.

11. Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek, and V. N. Kim. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO. J.* 23:4051-4060.

12. Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting, and G. J. Hannon. 2004. Processing of primary microRNAs by the Microprocessor complex. *Nature.* 432:231-235.

13. Gregory, R. I., K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, and R. Shiekhattar. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature.* 432:235-240.

14. Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin, and V. N. Kim. 2004. The Drosha-DGCR8 complex in primary microRNA processing. *Genes. Dev.* 18:3016-3027.

15. Landthaler, M., A. Yalcin, and T. Tuschl. 2004. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Curr. Biol.* 14:2162-2167.

16. Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, and V. N. Kim. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature.* 425:415-419.

17. Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg, and U. Kutay. 2004. Nuclear export of microRNA precursors. *Science.* 303:95-98.

18. Yi, R., Y. Qin, I. G. Macara, and B. R. Cullen. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes. Dev.* 17:3011-3016.

19. Zeng, Y., and B. R. Cullen. 2004. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic. Acids. Res.* 32:4776-4785.

20. Du, T., and P. D. Zamore. 2005. microPrimer: the biogenesis and function of microRNA. *Development.* 132:4645-4652.

21. Filipowicz, W., S. N. Bhattacharyya, and N. Sonenberg. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat. Rev. Genet.* 9:102-114.

22. Ruby, J. G., C. H. Jan, and D. P. Bartel. 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature.* 448:83-86.

23. Berezikov, E., W. J. Chung, J. Willis, E. Cuppen, and E. C. Lai. 2007. Mammalian mirtron genes. *Mol. Cell.* 28:328-336.

24. Okamura, K., J. W. Hagen, H. Duan, D. M. Tyler, and E. C. Lai. 2007. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell.* 130:89-100.

25. Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl. 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell.* 15:185-197.

26. Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, and G. J. Hannon. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. *Science.* 305:1437-1441.

27. Pillai, R. S., C. G. Artus, and W. Filipowicz. 2004. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA.* 10:1518-1525.

28. Eystathioy, T., E. K. L. Chan, S. A. Tenenbaum, J. D. Keene, K. Griffith, and M. J. Fritzler. 2002. A phosphorylated cytoplasmic autoantigen, GW182, associates with a

unique population of human mRNAs within novel cytoplasmic speckles. *Mol. Biol. Cell.* 13:1338-1351.

29. Bhanji, R. A., T. Eystathioy, E. K. L. Chan, D. B. Bloch, and M. J. Fritzler. 2007. Clinical and serological features of patients with autoantibodies to GW/P bodies. *Clin. Immunol.* 125:247-256.

30. Ingelfinger, D., D. J. Arndt-Jovin, R. Luhrmann, and T. Achsel. 2002. The human LSm1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. *RNA.* 8:1489-1501.

31. Sheth, U., and R. Parker. 2003. Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science.* 300:805-808.

32. van Dijk, E., N. Cougot, S. Meyer, S. Babajko, E. Wahle, and B. Seraphin. 2002. Human Dcp2: a catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO. J.* 21:6915-6924.

33. Yang, Z., A. Jakymiw, M. R. Wood, T. Eystathioy, R. L. Rubin, M. J. Fritzler, and E. K. L. Chan. 2004. GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. *J. Cell. Sci.* 117:5567-5578.

34. Jakymiw, A., S. Lian, T. Eystathioy, S. Li, M. Satoh, J. C. Hamel, M. J. Fritzler, and E. K. L. Chan. 2005. Disruption of GW bodies impairs mammalian RNA interference. *Nat. Cell. Biol.* 7:1267-1274.

35. Liu, J., M. A. Valencia-Sanchez, G. J. Hannon, and R. Parker. 2005. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat. Cell. Biol.* 7:719-723.

36. Pauley, K. M., T. Eystathioy, A. Jakymiw, J. C. Hamel, M. J. Fritzler, and E. K. L. Chan. 2006. Formation of GW bodies is a consequence of miRNA genesis. *EMBO. Rep.* 7:904-910.

37. Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand, and W. Filipowicz. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science.* 309:1573-1576.

38. Sen, G. L., and H. M. Blau. 2005. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat. Cell. Biol.* 7:633-636.

39. Lian, S., M. J. Fritzler, J. Katz, T. Hamazaki, N. Terada, M. Satoh, and E. K. L. Chan. 2007. Small Interfering RNA-mediated Silencing Induces Target-dependent Assembly of GW/P Bodies. *Mol. Biol. Cell.* 18:3375-3387.

40. Taganov, K. D., M. P. Boldin, K. J. Chang, and D. Baltimore. 2006. NF-B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. U S. A.* 103:12481-12486.

41. Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim, and D. P. Bartel. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol. Cell.* 27:91-105.

42. O'Connell, R. M., K. D. Taganov, M. P. Boldin, G. Cheng, and D. Baltimore. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. U S. A.* 104:1604-1609.

43. Tili, E., J. J. Michaille, A. Cimino, S. Costinean, C. D. Dumitru, B. Adair, M. Fabbri, H. Alder, C. G. Liu, G. A. Calin, and C. M. Croce. 2007. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J. Immunol.* 179:5082-5089.

44. O'Connell, R. M., D. S. Rao, A. A. Chaudhuri, M. P. Boldin, K. D. Taganov, J. Nicoll, R. L. Paquette, and D. Baltimore. 2008. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* 205:585-594.

45. Chen, X. M., P. L. Splinter, S. P. O'hara, and N. F. Larusso. 2007. A cellular miRNA, let-7i, regulates toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. *J. Biol. Chem.* 282:28929-28938.

46. Tam, W., D. Ben-Yehuda, and W. S. Hayward. 1997. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol. Cell. Biol.* 17:1490-1502.

47. Tam, W. 2001. Identification and characterization of human BIC, a gene on chromosome 21 that encodes a noncoding RNA. *Gene.* 274:157-167.

48. Haasch, D., Y. W. Chen, R. M. Reilly, X. G. Chiou, S. Koterski, M. L. Smith, P. Kroeger, K. McWeeny, D. N. Halbert, K. W. Mollison, S. W. Djuric, and J. M. Trevillyan. 2002. T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. *Cell. Immunol.* 217:78-86.

49. van den Berg, A., B. J. Kroesen, K. Kooistra, D. de Jong, J. Briggs, T. Blokzijl, S. Jacobs, J. Kluiver, A. Diepstra, E. Maggio, and S. Poppema. 2003. High expression of B-cell receptor inducible gene BIC in all subtypes of Hodgkin lymphoma. *Genes. Chromosomes. Cancer.* 37:20-28.

50. Costinean, S., N. Zanesi, Y. Pekarsky, E. Tili, S. Volinia, N. Heerema, and C. M. Croce. 2006. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc. Natl. Acad. Sci. U S. A.* 103:7024-7029.

51. Kluiver, J., S. Poppema, D. de Jong, T. Blokzijl, G. Harms, S. Jacobs, B. J. Kroesen, and A. van den Berg. 2005. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J. Pathol.* 207:243-249.

52. Metzler, M., M. Wilda, K. Busch, S. Viehmann, and A. Borkhardt. 2004. High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes. Chromosomes. Cancer.* 39:167-169.

53. Rodriguez, A., E. Vigorito, S. Clare, M. V. Warren, P. Couttet, D. R. Soond, S. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, D. Vetrie, K. Okkenhaug, A. J. Enright, G. Dougan, M. Turner, and A. Bradley. 2007. Requirement of bic/microRNA-155 for normal immune function. *Science.* 316:608-611.

54. Thai, T. H., D. P. Calado, S. Casola, K. M. Ansel, C. Xiao, Y. Xue, A. Murphy, D. Frendewey, D. Valenzuela, J. L. Kutok, M. Schmidt-Supprian, N. Rajewsky, G. Yancopoulos, A. Rao, and K. Rajewsky. 2007. Regulation of the germinal center response by microRNA-155. *Science.* 316:604-608.

55. Vigorito, E., K. L. Perks, C. breu-Goodger, S. Bunting, Z. Xiang, S. Kohlhaas, P. P. Das, E. A. Miska, A. Rodriguez, A. Bradley, K. G. Smith, C. Rada, A. J. Enright, K. M. Toellner, I. C. Maclennan, and M. Turner. 2007. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity.* 27:847-859.

56. Neilson, J. R., G. X. Zheng, C. B. Burge, and P. A. Sharp. 2007. Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes. Dev.* 21:578-589.

57. Wu, H., J. R. Neilson, P. Kumar, M. Manocha, P. Shankar, P. A. Sharp, and N. Manjunath. 2007. miRNA Profiling of Naive, Effector and Memory CD8 T Cells. *PLoS. ONE.* 2:e1020.

58. Zhou, B., S. Wang, C. Mayr, D. P. Bartel, and H. F. Lodish. 2007. miR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. *Proc. Natl. Acad. Sci. U S. A.* 104:7080-7085.

59. Chen, C. Z., L. Li, H. F. Lodish, and D. P. Bartel. 2004. MicroRNAs modulate hematopoietic lineage differentiation. *Science.* 303:83-86.

60. Li, Q. J., J. Chau, P. J. Ebert, G. Sylvester, H. Min, G. Liu, R. Braich, M. Manoharan, J. Soutschek, P. Skare, L. O. Klein, M. M. Davis, and C. Z. Chen. 2007. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell.* 129:147-161.

61. Fazi, F., A. Rosa, A. Fatica, V. Gelmetti, M. L. De Marchis, C. Nervi, and I. Bozzoni. 2005. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell.* 123:819-831.

62. Fukao, T., Y. Fukuda, K. Kiga, J. Sharif, K. Hino, Y. Enomoto, A. Kawamura, K. Nakamura, T. Takeuchi, and M. Tanabe. 2007. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell.* 129:617-631.

63. Xiao, C., D. P. Calado, G. Galler, T. H. Thai, H. C. Patterson, J. Wang, N. Rajewsky, T. P. Bender, and K. Rajewsky. 2007. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell.* 131:146-159.

64. Lecellier, C. H., P. Dunoyer, K. Arar, J. Lehmann-Che, S. Eyquem, C. Himber, A. Saib, and O. Voinnet. 2005. A cellular microRNA mediates antiviral defense in human cells. *Science.* 308:557-560.

65. Pedersen, I. M., G. Cheng, S. Wieland, S. Volinia, C. M. Croce, F. V. Chisari, and M. David. 2007. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature.* 449:919-922.

66. Jopling, C. L., M. Yi, A. M. Lancaster, S. M. Lemon, and P. Sarnow. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science.* 309:1577-1581.

67. Cai, X., S. Lu, Z. Zhang, C. M. Gonzalez, B. Damania, and B. R. Cullen. 2005. Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U S. A.* 102:5570-5575.

68. Pfeffer, S., A. Sewer, M. Lagos-Quintana, R. Sheridan, C. Sander, F. A. Grasser, L. F. van Dyk, C. K. Ho, S. Shuman, M. Chien, J. J. Russo, J. Ju, G. Randall, B. D. Lindenbach, C. M. Rice, V. Simon, D. D. Ho, M. Zavolan, and T. Tuschl. 2005. Identification of microRNAs of the herpesvirus family. *Nat. Methods.* 2:269-276.

69. Samols, M. A., J. Hu, R. L. Skalsky, and R. Renne. 2005. Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79:9301-9305.

70. Samols, M. A., R. L. Skalsky, A. M. Maldonado, A. Riva, M. C. Lopez, H. V. Baker, and R. Renne. 2007. Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS. Pathog.* 3:e65.

71. Skalsky, R. L., M. A. Samols, K. B. Plaisance, I. W. Boss, A. Riva, M. C. Lopez, H. V. Baker, and R. Renne. 2007. Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* 81:12836-12845.

72. Pfeffer, S., M. Zavolan, F. A. Grasser, M. Chien, J. J. Russo, J. Ju, B. John, A. J. Enright, D. Marks, C. Sander, and T. Tuschl. 2004. Identification of virus-encoded microRNAs. *Science.* 304:734-736.

73. Stern-Ginossar, N., N. Elefant, A. Zimmermann, D. G. Wolf, N. Saleh, M. Biton, E. Horwitz, Z. Prokocimer, M. Prichard, G. Hahn, D. Goldman-Wohl, C. Greenfield, S. Yagel, H. Hengel, Y. Altuvia, H. Margalit, and O. Mandelboim. 2007. Host immune system gene targeting by a viral miRNA. *Science.* 317:376-381.

74. Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J. P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini, and C. M. Croce. 2005. MicroRNA gene expression deregulation in human breast cancer. *Cancer. Res.* 65:7065-7070.

75. Volinia, S., G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris, and C. M. Croce. 2006. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. U S. A.* 103:2257-2261.

76. Yanaihara, N., N. Caplen, E. Bowman, M. Seike, K. Kumamoto, M. Yi, R. M. Stephens, A. Okamoto, J. Yokota, T. Tanaka, G. A. Calin, C. G. Liu, C. M. Croce, and C. C. Harris. 2006. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer. Cell.* 9:189-198.

77. Martin, M. M., E. J. Lee, J. A. Buckenberger, T. D. Schmittgen, and T. S. Elton. 2006. MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts. *J. Biol. Chem.* 281:18277-18284.

78. Eis, P. S., W. Tam, L. Sun, A. Chadburn, Z. Li, M. F. Gomez, E. Lund, and J. E. Dahlberg. 2005. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc. Natl. Acad. Sci. U S. A.* 102:3627-3632.

79. Calin, G. A., C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich, and C. M. Croce. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U S. A.* 99:15524-15529.

80. Cimmino, A., G. A. Calin, M. Fabbri, M. V. Iorio, M. Ferracin, M. Shimizu, S. E. Wojcik, R. I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini, and C. M. Croce. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl. Acad. Sci. U S. A.* 102:13944-13949.

81. Tanzer, A., and P. F. Stadler. 2004. Molecular evolution of a microRNA cluster. *J. Mol. Biol.* 339:327-335.

82. He, L., J. M. Thomson, M. T. Hemann, E. Hernando-Monge, D. Mu, S. Goodson, S. Powers, C. Cordon-Cardo, S. W. Lowe, G. J. Hannon, and S. M. Hammond. 2005. A microRNA polycistron as a potential human oncogene. *Nature.* 435:828-833.

83. Venturini, L., K. Battmer, M. Castoldi, B. Schultheis, A. Hochhaus, M. U. Muckenthaler, A. Ganser, M. Eder, and M. Scherr. 2007. Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. *Blood.* 109:4399-4405.

84. Xiao, C., L. Srinivasan, D. P. Calado, H. C. Patterson, B. Zhang, J. Wang, J. M. Henderson, J. L. Kutok, and K. Rajewsky. 2008. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat. Immunol.* 9:405-414.

85. Akao, Y., Y. Nakagawa, and T. Naoe. 2006. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol. Pharm. Bull.* 29:903-906.

86. Johnson, S. M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K. L. Reinert, D. Brown, and F. J. Slack. 2005. RAS is regulated by the let-7 microRNA family. *Cell.* 120:635-647.

87. Takamizawa, J., H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, T. Harano, Y. Yatabe, M. Nagino, Y. Nimura, T. Mitsudomi, and T. Takahashi. 2004. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer. Res.* 64:3753-3756.

88. Yu, F., H. Yao, P. Zhu, X. Zhang, Q. Pan, C. Gong, Y. Huang, X. Hu, F. Su, J. Lieberman, and E. Song. 2007. let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell.* 131:1109-1123.

89. Abelson, J. F., K. Y. Kwan, B. J. O'Roak, D. Y. Baek, A. A. Stillman, T. M. Morgan, C. A. Mathews, D. L. Pauls, M. R. Rasin, M. Gunel, N. R. Davis, A. G. Ercan-Sencicek, D. H. Guez, J. A. Spertus, J. F. Leckman, L. S. Dure, R. Kurlan, H. S. Singer, D. L. Gilbert, A. Farhi, A. Louvi, R. P. Lifton, N. Sestan, and State MW. 2005. Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science.* 310:317-320.

90. Dai, Y., Y. S. Huang, M. Tang, T. Y. Lv, C. X. Hu, Y. H. Tan, Z. M. Xu, and Y. B. Yin. 2007. Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus.* 16:939-946.

91. Lukiw, W. J. 2007. Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport.* 18:297-300.

92. Yu, D., A. H. Tan, X. Hu, V. Athanasopoulos, N. Simpson, D. G. Silva, A. Hutloff, K. M. Giles, P. J. Leedman, K. P. Lam, C. C. Goodnow, and C. G. Vinuesa. 2007. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature.* 450:299-303.

93. Satoh, M., J. J. Langdon, C. H. Chou, D. P. McCauliffe, E. L. Treadwell, T. Ogasawara, M. Hirakata, A. Suwa, P. L. Cohen, R. A. Eisenberg, and . 1994. Characterization of the Su antigen, a macromolecular complex of 100/102 and 200-kDa proteins recognized by autoantibodies in systemic rheumatic diseases. *Clin. Immunol. Immunopathol.* 73:132-141.

94. Jakymiw, A., K. Ikeda, M. J. Fritzler, W. H. Reeves, M. Satoh, and E. K. L. Chan. 2006. Autoimmune targeting of key components of RNA interference. *Arthritis. Res. Ther.* 8:R87.

95. Yu, J. H., W. H. Yang, T. Gulick, K. D. Bloch, and D. B. Bloch. 2005. Ge-1 is a central component of the mammalian cytoplasmic mRNA processing body. *RNA.* 11:1795-1802.

96. Liu, J., F. V. Rivas, J. Wohlschlegel, J. R. Yates, III, R. Parker, and G. J. Hannon. 2005. A role for the P-body component GW182 in microRNA function. *Nat. Cell. Biol.* 7:1261-1266.

97. Meister, G., M. Landthaler, L. Peters, P. Y. Chen, H. Urlaub, R. Luhrmann, and T. Tuschl. 2005. Identification of novel argonaute-associated proteins. *Curr. Biol.* 15:2149-2155.

98. Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz. 2006. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell.* 125:1111-1124.

99. Vasudevan, S., Y. Tong, and J. A. Steitz. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science.* 318:1931-1934.

100. Teixeira, D., U. Sheth, M. A. Valencia-Sanchez, M. Brengues, and R. Parker. 2005. Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA.* 11:371-382.

101. Cougot, N., S. Babajko, and B. Seraphin. 2004. Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell. Biol.* 165:31-40.

102. Ding, L., A. Spencer, K. Morita, and M. Han. 2005. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in C. elegans. *Mol. Cell.* 19:437-447.

103. Andrei, M. A., D. Ingelfinger, R. Heintzmann, T. Achsel, R. Rivera-Pomar, and R. Luhrmann. 2005. A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. *RNA.* 11:717-727.

104. Eystathioy, T., A. Jakymiw, E. K. L. Chan, B. Seraphin, N. Cougot, and M. J. Fritzler. 2003. The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA.* 9:1171-1173.

105. Bashkirov, V. I., H. Scherthan, J. A. Solinger, J. M. Buerstedde, and W. D. Heyer. 1997. A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *J. Cell. Biol.* 136:761-773.

106. Fukuhara, N., J. Ebert, L. Unterholzner, D. Lindner, E. Izaurralde, and E. Conti. 2005. SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA decay pathway. *Mol. Cell.* 17:537-547.

107. Kedersha, N., G. Stoecklin, M. Ayodele, P. Yacono, J. Lykke-Andersen, M. J. Fritzler, D. Scheuner, R. J. Kaufman, D. E. Golan, and P. Anderson. 2005. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *J. Cell. Biol.* 169:871-884.

108. Unterholzner, L., and E. Izaurralde. 2004. SMG7 acts as a molecular link between mRNA surveillance and mRNA decay. *Mol. Cell.* 16:587-596.

109. Rehwinkel, J., I. Behm-Ansmant, D. Gatfield, and E. Izaurralde. 2005. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA.* 11:1640-1647.

110. Tang, G. 2005. siRNA and miRNA: an insight into RISCs. *Trends. Biochem. Sci.* 30:106-114.

111. Tomari, Y., and P. D. Zamore. 2005. MicroRNA biogenesis: drosha can't cut it without a partner. *Curr. Biol.* 15:R61-R64.

112. Valencia-Sanchez, M. A., J. Liu, G. J. Hannon, and R. Parker. 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes. Dev.* 20:515-524.

113. Fenger-Gron, M., C. Fillman, B. Norrild, and J. Lykke-Andersen. 2005. Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol. Cell.* 20:905-915.

114. Eulalio, A., I. Behm-Ansmant, and E. Izaurralde. 2007. P bodies: at the crossroads of post-transcriptional pathways. *Nat. Rev. Mol. Cell. Biol.* 8:9-22.

115. Jakymiw, A., K. M. Pauley, S. Li, K. Ikeda, S. Lian, T. Eystathioy, M. Satoh, M. J. Fritzler, and E. K. L. Chan. 2007. The role of GW/P-bodies in RNA processing and silencing. *J. Cell. Sci.* 120:1317-1323.

116. Eulalio, A., I. Behm-Ansmant, D. Schweizer, and E. Izaurralde. 2007. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol. Cell. Biol.* 27:3970-3981.

117. Khabar, K. S. 2005. The AU-rich transcriptome: more than interferons and cytokines, and its role in disease. *J. Interferon. Cytokine. Res.* 25:1-10.

118. Jing, Q., S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, P. F. Di, S. C. Lin, H. Gram, and J. Han. 2005. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell.* 120:623-634.

119. Takahashi, H., M. Maeda, H. Sawa, H. Hasegawa, M. Moriyama, T. Sata, W. W. Hall, and T. Kurata. 2006. Dicer and positive charge of proteins decrease the stability of RNA containing the AU-rich element of GM-CSF. *Biochem. Biophys. Res. Commun.* 340:807-814.

120. Gantier, M. P., A. J. Sadler, and B. R. Williams. 2007. Fine-tuning of the innate immune response by microRNAs. *Immunol. Cell. Biol.* 85:458-462.

121. Taganov, K. D., M. P. Boldin, and D. Baltimore. 2007. MicroRNAs and immunity: tiny players in a big field. *Immunity.* 26:133-137.

122. Guha, M., and N. Mackman. 2001. LPS induction of gene expression in human monocytes. *Cell. Signal.* 13:85-94.

123. Carpenter, A. E., T. R. Jones, M. R. Lamprecht, C. Clarke, I. H. Kang, O. Friman, D. A. Guertin, J. H. Chang, R. A. Lindquist, J. Moffat, P. Golland, and D. M. Sabatini. 2006. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome. Biol.* 7:R100.

124. Griffith, K. J., E. K. L. Chan, C. C. Lung, J. C. Hamel, X. Guo, K. Miyachi, and M. J. Fritzler. 1997. Molecular cloning of a novel 97-kd Golgi complex autoantigen associated with Sjogren's syndrome. *Arthritis. Rheum.* 40:1693-1702.

125. Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image Processing with ImageJ. *Biophotonics International* 11:36-42.

126. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25:402-408.

127. Chu, C. Y., and T. M. Rana. 2006. Translation Repression in Human Cells by MicroRNA-Induced Gene Silencing Requires RCK/p54. *PLoS. Biol.* 4:e210.

128. Franks, T. M., and J. Lykke-Andersen. 2007. TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements. *Genes. Dev.* 21:719-735.

129. Bakheet, T., B. R. Williams, and K. S. Khabar. 2006. ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic. Acids. Res.* 34:D111-D114.

130. Bakheet, T., M. Frevel, B. R. Williams, W. Greer, and K. S. Khabar. 2001. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic. Acids. Res.* 29:246-254.

131. Griffiths-Jones S. 2006. miRBase: the microRNA sequence database. *Methods. Mol. Biol.* 342:129-138.

132. Smolen, J. S., D. Aletaha, M. Koeller, M. H. Weisman, and P. Emery. 2007. New therapies for treatment of rheumatoid arthritis. *Lancet.* 370:1861-1874.

133. Bresnihan, B., J. M. varo-Gracia, M. Cobby, M. Doherty, Z. Domljan, P. Emery, G. Nuki, K. Pavelka, R. Rau, B. Rozman, I. Watt, B. Williams, R. Aitchison, D. McCabe, and P. Musikic. 1998. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis. Rheum.* 41:2196-2204.

134. Lipsky, P. E., D. M. van der Heijde, E. W. St Clair, D. E. Furst, F. C. Breedveld, J. R. Kalden, J. S. Smolen, M. Weisman, P. Emery, M. Feldmann, G. R. Harriman, and R. N. Maini. 2000. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N. Engl. J. Med.* 343:1594-1602.

135. Fabbri, M., C. M. Croce, and G. A. Calin. 2008. MicroRNAs. *Cancer. J.* 14:1-6.

136. Lodish, H. F., B. Zhou, G. Liu, and C. Z. Chen. 2008. Micromanagement of the immune system by microRNAs. *Nat. Rev. Immunol.* 8:120-130.

137. Nakasa, T., S. Miyaki, A. Okubo, M. Hashimoto, K. Nishida, M. Ochi, and H. Asahara. 2008. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis. Rheum.* 58:1284-1292.

138. Stanczyk, J., D. M. Pedrioli, F. Brentano, O. Sanchez-Pernaute, C. Kolling, R. E. Gay, M. Detmar, S. Gay, and D. Kyburz. 2008. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis. Rheum.* 58:1001-1009.

139. Cuschieri, J., V. Bulmus, D. Gourlay, I. Garcia, A. Hoffman, P. Stayton, and R. V. Maier. 2004. Modulation of macrophage responsiveness to lipopolysaccharide by IRAK-1 manipulation. *Shock.* 21:182-188.

140. Pauley, K. M., M. Satoh, P. R. Dominguez-Gutierrez, S. M. Wallet, L. S. Holliday, W. H. Reeves, and E. K. L. Chan. 2008. Formation of GW/P Bodies as Marker for miRNA-Mediated Regulation of Innate Immune Signaling. *Mol. Biol. Cell.* submitted.

141. Broad, A., D. E. Jones, and J. A. Kirby. 2006. Toll-like receptor (TLR) response tolerance: a key physiological "damage limitation" effect and an important potential opportunity for therapy. *Curr. Med. Chem.* 13:2487-2502.

142. Li, Y., E. A. Howell, A. S. Lagoo, M. Kuchibhatla, H. Pan, H. J. Cohen, and S. A. Lagoo. 2008. Differential gene expression of IRAK-1 and IRAK-M in peripheral blood mononuclear cells of young and aged rats following preconditioning with endotoxin. *Shock.*

BIOGRAPHICAL SKETCH

Kaleb Marie Pauley was born in Charleston, West Virginia. She graduated from South Charleston High School in 1998, and then attended college at Morehead State University in Morehead, Kentucky for two years. In 2000, she transferred to Marshall University in Huntington, West Virginia where she earned a Bachelor in Science degree in biological sciences in 2002. At this time, Kaleb developed an interest in research and decided to pursue a Master of Science degree in biological sciences in the laboratory of Dr. Laura Jenski. With Dr. Jenski's guidance, Kaleb managed the flow cytometry core facility while she completed her research project focused on the role of docosahexeanoic acid in modulating class I major histocompatibility complex structure and function. During this time, Kaleb decided to continue her research career and was accepted to the Interdisciplinary Program in biomedical sciences at the University of Florida. In the summer of 2004, Kaleb defended her master's thesis, married the love of her life, and moved to Gainesville, Florida to pursue her Ph.D.

Kaleb decided to join the immunology and microbiology concentration, and ended up joining the lab of Dr. Edward K.L. Chan. Here, her research focused on the role of microRNA regulation in GW/P body formation, innate immune signaling, and rheumatoid arthritis. After her doctorate work, Kaleb plans on accepting a postdoctoral position in an immunology lab where she can widen her knowledge of autoimmune diseases. She also hopes to start a family with her husband, Brad.