THE FUNCTION OF GW182 AND GW/P BODY IN RNA INTERFERENCE

By

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To my parents who have inspired and supported me to pursue my dream, to my mentor who has guided me through adversities and frustrations, to my husband who supports me every day.
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RNA interference (RNAi) is an important mechanism that regulates gene expression and is conserved from plant to human. In this mechanism, small interfering RNAs (siRNAs) or microRNAs (miRNAs) specifically regulate the stability and translation of mRNA with complementary sequence in the encoding region or in the 3’-UTR. RNAi has become an important technique that is broadly adopted to study gene function in research laboratories and is also a new therapeutic strategy for a variety of diseases including viral infections, genetic diseases, and cancers. In our study, we discover that GW/P bodies (GWB), distinct cytoplasmic foci for mRNA degradation, are processing centers for mRNAs targeted by RNAi pathway. Knockdown of GW182, a GWB marker important for body formation, prevented the localization of Ago2, a key enzyme in RNAi pathway, to cytoplasmic foci and greatly impaired siRNA-mediated silencing. It suggested that GW182 and/or GWB were indispensable for RNAi process. Moreover, the number and size of GWB greatly increase when siRNA with a cellular target is introduced into mammalian cells. The increase of GWB correlated with siRNA-mediated silencing. Knockdown of GW182 or Ago2 greatly impaired RNAi function and abolished the siRNA-induced increase of GWB. Therefore, RNAi activity promotes the
assembly of GWB which can potentially serve as cellular markers for monitoring RNAi activity during therapy. Furthermore, we reveal that GW182 is a possible suppressor in the miRNA-mediated silencing. GW182 interacts with human Ago1-4 proteins, the core components of RNAi silencing effector complex. Interestingly, several non-overlapping regions of GW182 bind to the C-terminal half of Ago2 independently suggesting that GW182 may interact with multiple Ago proteins simultaneously. Moreover, the GW182-Ago interaction may recruit Ago2-miRNAs-mRNA complex to GWB for processing which contributes to the formation of GWB. Most importantly, tethering Ago2 or the C-terminal half of Ago2 to the 3’-UTR caused translational repression which required GW182. This implicated that GW182 was possibly the repressor brought to the 3’-UTR by Ago2 to switch off translation. Taken together, our study has advanced the understanding of the molecular and cell biology of RNAi and may potentially provide insight into future application and monitor of RNAi.
Chapter 1
Introduction

GW182 and GW Body

In 2002, GW182 is identified and cloned by our laboratory as a novel autoantigen using autoimmune serum from a patient with motor and sensory neuropathy. GW182 is a 182 kDa protein with a classical RNA recognition motif at its C-terminus and is characterized by multiple glycine/tryptophan (GW) repeats. GW182 is found to be associated with a specific subset of mRNAs and consistently reside within unique cytoplasmic foci designated as GW bodies that are distinct from other known cytoplasmic organelles such as Golgi complex, endosomes, lysosomes or peroxisomes (Eystathioy et al., 2002a). It is speculated that GW bodies are involved in the post-transcriptional regulation of gene expression by sequestering a subset of gene transcripts involved in cell growth and homeostasis. GW bodies are small, generally spherical, cytoplasmic foci that vary in number and size at different stages of the cell cycle (Yang et al., 2004). Electron microscopy demonstrates that GW bodies are electron dense structures of 100–300 nm in diameter devoid of a lipid bilayer membrane. These structures comprise of clusters of electron dense strands of 8–10 nm in diameter. In vitro gene knockdown of GW182 using short hairpin RNA (shRNA) plasmid results in instability and disappearance of GW bodies (Yang et al., 2004). Autoantibodies to GW182/GW bodies are typically found in patients with Sjögren’s syndrome, mixed motor/sensory neuropathy, and systemic lupus erythematosus (Eystathioy et al., 2003a;Bhanji et al., 2007).

GW/P Bodies Are Cytoplasmic Sites for Messenger RNA Decay

In the past decade, studies have shown that the 5’-3’ mRNA degradation factors, including Xrn1 (5’-3’ exonuclease), Dcp2:Dcp1 (decapping enzyme), and LSm1-7 complex (stimulator of mRNA decapping), colocalize in distinct cytoplasmic foci (Heyer et al., 1995;Bashkirov et al.,
1997; van Dijk et al., 2002; Ingelfinger et al., 2002). However, the function of these cytoplasmic foci is unclear. In 2003, Sheth and Parker report that in yeast these cytoplasmic foci contain mRNA degradation intermediates in addition to the 5′-3′ mRNA decay factors and designate the foci as processing bodies (P bodies) (Sheth and Parker, 2003). They propose that P bodies are dynamic sites involved in the regulation of mRNA degradation and storage, and that the flux of mRNAs between polysomes and P bodies are a critical aspect of cytoplasmic mRNAs metabolism. In mammalian cells, decay factors Dcp1 and LSm4 co-localize with GW182 in GWB, which is shown to contain poly (A)⁺ RNA and dynamically disappear as mRNA breakdown was abolished (Eystathioy et al., 2003b; Cougot et al., 2004). Therefore, GWB are considered as the mammalian analogues of P bodies and as the sites for active 5′-3′ mRNA degradation, which are designated here provisionally as GW/P bodies (GWB).

In addition to the 5′-3′ mRNA decay pathway, GWB are also considered sites for the nonsense-mediated decay (NMD) and AU-rich element (ARE)-mediated decay pathways. NMD is an mRNA quality control mechanism that degrades aberrant mRNAs having a premature translational termination codon (PTC), thereby preventing the synthesis of truncated and potentially harmful proteins (Conti and Izaurralde, 2005). Dcp1:Dcp2 decapping complex is shown to be associated with Upf1, a component central to NMD (Muhlrad and Parker, 1994; He and Jacobson, 1995; Lykke-Andersen, 2002), and decapped mRNAs in NMD. Depletion of the decapping complex subunit Dcp2 results in impaired NMD (Lejeune et al., 2003). Inhibition of NMD reveals that mRNA and NMD factors are dynamically and sequentially recruited to GWB (Durand et al., 2007). AU-rich elements (AREs) are found in the 3′ untranslated region (3′-UTR) of a variety of short-lived mRNAs in mammalian cells. The human Dcp1:Dcp2 complex and other mRNA decay enzymes recruit ARE-containing mRNAs via Tristetraprolin (TTP), the
ARE-binding protein that activates mRNA decay (Lykke-Andersen and Wagner, 2005). TTP interacts with Dcp2 and decapping activator Edc3 to activate decapping (Fenger-Gron et al., 2005; Franks and Lykke-Andersen, 2007). In addition, TTP and its paralog BRF-1 could nucleate GWB formation to silence ARE-mRNAs (Franks and Lykke-Andersen, 2007).

**RNA Interference Is Linked to GW/P Body and GW182**

**RNA Interference**

RNA interference (RNAi) is initially described in plants as a genetic control mechanism implicated in virus resistance (Ratcliff et al., 1997; Covey et al., 1997), genome maintenance (Assaad et al., 1993) and developmental control (Boerjan et al., 1994). This mechanism is further characterized in C. elegans as a potent and sequence-specific mechanism that silences endogenous genes (Fire et al., 1998). Based on up-to-date studies, RNAi includes siRNA- and miRNA-mediated silencing. In siRNA-mediated silencing, the dsRNAs, which are formed in cells or are introduced into cells by viral infection or artificial expression, are processed by RNase III enzyme, Dicer, into ~20-bp double-stranded small interfering RNAs (siRNAs). The siRNAs are then unwound and the antisense strands are incorporated into RNA-induced silencing complex (RISC). Subsequently, RISC binds to and cleaves target mRNA with complete complementary sequence to the siRNA (Filipowicz et al., 2005). Argonaute 2 (Ago2) is the core component of RISC and harbors RNase H activity responsible for the cleavage of target mRNA (Liu et al., 2004).

MicroRNAs (miRNAs) are endogenous ~21-nt regulatory RNAs that are evolutionarily conserved in most species and are estimated to regulate ~30% of protein-encoding genes in human (Lewis et al., 2005). MiRNAs are processed from endogenous precursor molecules folded into hairpin-like structure. The maturation of miRNAs includes two steps, both catalyzed by enzymes of the RNase III family, Drosha and Dicer. Drosha is responsible for the processing
of primary miRNA transcripts (pri-miRNAs) to ~70-nt hairpins named precursor miRNAs (pre-
miRNAs). Subsequently, Dicer processes pre-miRNAs into mature miRNAs, which binds to the
3’-UTR of target mRNA with imperfect complementary sequence and regulates gene expression
by increasing instability or repressing translation of target mRNA (Filipowicz et al., 2005). Like
siRNA, miRNA also forms RISC-like ribonucleoprotein particles (miRNPs). Argonaute proteins
are the core components of both RISC and miRNPs.

**GW/P Body and GW182 Are Closely Correlated with siRNA and miRNA Silencing
Function**

As a potentially powerful tool for experimental gene knockdown and clinical therapy,
RNAi has been extensively studied over the past decade. However, many of the studies on
RNAi have only used biochemical techniques with little attention to the cell biology of RNAi.
Recently evidence have linked mRNA turnover to RNAi at the level of cell biology.
Exonucleases AtXrn4 (A. thaliana) and dXrn1 (D. melanogaster), whose mammalian orthologue
Xrn1 is a GWB component (Cougot et al., 2004), are demonstrated to be required for
degradation of the 3’ fragment of RISC-targeted mRNA. Knockout of AtXrn4 or dXrn1-
knockdown by siRNAs results in accumulation of the 3’ fragment of the cleaved mRNA
(Gazzani et al., 2004; Souret et al., 2004; Orban and Izaurralde, 2005). Interestingly, further
studies show that all human Ago proteins localize to GWB and that Ago2 interacts with GWB
components Dcp2 and Xrn1 (Sen and Blau, 2005; Pillai et al., 2005; Liu et al., 2005b). In
addition, miRNAs and their target mRNAs are present in GWB and the target mRNAs localize to
the foci in a miRNA-dependent manner (Pillai et al., 2005; Liu et al., 2005b).

We are one of the first groups to describe the link between RNAi and GWB/GW182. Two
studies from our laboratory strongly support this correlation. The first study focusing on siRNA-
mediated function shows that transfected Cy3-labeled siRNAs are present in GWB and could be
immunoprecipitated by anti-GWB serum. More importantly, disruption of GWB by dominant interfering mutants significantly impairs siRNA silencing function (Jakymiw et al., 2005). The other study focusing on miRNA-mediated function shows that, similarly, transfected Cy3-labeled let-7 miRNAs localize to GWB and could be detected in the immunoprecipitate using an anti-GWB serum. Moreover, knockdown of Drosha or DGCR8, the two factors forming the microprocessor complex to mediate the biogenesis of miRNAs, leads to a blockage in the maturation of miRNAs and disassembly of GWB. Transfecting siRNA into the Drosha-knockdown cells reassembles GWB (Pauley et al., 2006). In addition to siRNA/miRNA, we demonstrate that the key factor of RNAi, Ago2, interacts with GW182 suggesting that GW182 could be important for siRNA- and miRNA-mediated function in human cells. Studies of GW182 orthologs in Drosophila and C. elegans strongly support this. Knockdown of the Drosophila GW182 ortholog greatly impairs miRNA-mediated gene silencing activity in Drosophila cells (Rehwinkel et al., 2005). The C. elegans GW182 ortholog, AIN-1, is capable of interacting with an Ago protein and Dicer, and may target the Ago protein to cytoplasmic GW/P bodies in C. elegans (Ding et al., 2005). With all these evidence, we further characterize the function of GW182 and GWB in RNAi in the current study. We propose a model in which GWB are cytoplasmic processing centers for the mRNAs targeted by RNAi pathway with GW182 playing an important role in the pathway.
CHAPTER 2
KNOCKDOWN OF GW182 DISRUPTS GW/P BODY AND IMPAIRS RNA INTERFERENCE

Introduction

RNA interference (RNAi) is an efficient mechanism for post-transcriptional, sequence-specific regulation of gene expression and is conserved from plants to mammalian cells. Much research has been performed to gain a better understanding of RNAi from a biochemical point of view in the past few years, but little is known about intracellular location of RNAi activity. Only recently has the evidence been reported linking RNAi to GW/P bodies (GWB). One of the evidence is that exonuclease AtXrn4 (A. thaliana) or dXrn1 (D. melanogaster) are required for degradation of 3’ fragment of RISC-targeted mRNA (Gazzani et al., 2004; Souret et al., 2004; Orban and Izaurralde, 2005). In addition, Ago2, a key factor in RNAi, localizes to GWB and interacts with GW182 in mammalian cells (Sen and Blau, 2005; Jakymiw et al., 2005; Liu et al., 2005b). The localization of Ago2 is not altered by the presence or absence of siRNAs or their target mRNAs (Sen and Blau, 2005). Moreover, siRNAs, miRNAs and the mRNA targets are presence in GWB (Pillai et al., 2005; Jakymiw et al., 2005; Liu et al., 2005b). All these intriguing observations indicate that certain stage along RNAi or miRNA pathway occurs in GWB and prompt us to ask the following questions: (a) How GWB participate in RNAi? (b) What is the effect of disruption of GWB on RNAi activity? Studies from our laboratory has demonstrated that expression of the N-terminal 1/3 fragment of GW182 or the C-terminal 1/2 fragment of Ago2 is able to disrupt endogenous GWB formation and impair the silencing capability of siRNA to lamin A/C (Jakymiw et al., 2005). These results suggest that the GW182 protein and/or the microenvironment of GWB may contribute to the RISC and RNAi activity. However, the mechanisms on how these constructs disrupt GWB remain puzzling, which create uncertainty of the effect of GWB on RNAi. In this study, to further prove the importance of
GW182 for RNAi function, siRNA for GW182 is employed as a different method to disrupt GWB. The effect of siRNA for GW182 on RNAi activity is measured by the expression of endogenous lamin A/C.

**Materials and Methods**

**Antibodies**

The human prototype anti-GW182 and anti-hAgo2 sera were obtained from serum banks at the Advanced Diagnostics Laboratory, University of Calgary and the Division of Rheumatology and Clinical Immunology, University of Florida. Prototype human anti-GW182 serum was described previously (Yang et al., 2004). Rabbit anti-LSm4 polyclonal antibody was produced as described (Eystathioy et al., 2002b). Mouse monoclonal anti-lamin A/C 636 and anti-tubulin were purchased from Santa Cruz Biotechnology, Inc. and Sigma-Aldrich, respectively.

**Small Interfering RNA Synthesis**

The lamin A/C-siRNA targeting region was selected from a 21-nt target mRNA (position 608–628 relative to the start codon). The Cy3-5’-end labeled antisense and sense lamin siRNA duplex (5’Cy3-UGU UCU UCU GGA AGU CCA GdTdT3’ and 5’P-CUG GAC UUC CAG AAG AAC AdTdT3’, respectively) was chemically synthesized by Dharmacon. The Cy3-labeled Luciferase GL2 siRNA duplex and siGENOME SMARTpool reagent human TNRC6 (GW182) siRNA duplex were purchased from Dharmacon. The aforementioned duplexes were resuspended in 1x siRNA Universal buffer and the resulting 20 μM stock was stored in aliquots at -20°C prior to use.

**Human Cell Culture and siRNA Transfection**

HeLa cells were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO₂. HeLa cells were grown on coverslips to 30-40% confluency in a 6-well plate. The following day the media was
replaced with 1 ml of Opti-MEM I Reduced Serum Medium (Invitrogen) containing 10% FBS before transfection. In single siRNA transfection, 100 nM or 200 nM siRNAs were transiently transfected into HeLa cells using 7µL of Oligofectamine (Invitrogen). In siRNA co-transfection, 100 nM siRNA for GW182 were mixed with 100 nM siRNA for lamin A/C and were cotransfected into HeLa cells using 7µL of Oligofectamine. In siRNA sequential transfection, 200 nM or 40 nM siRNA for either GW182 or luciferase were transfected into cells using 7µL of Oligofectamine. 24 hours after the 1st transfection, the media was replaced with 1 ml of Opti-MEM I Reduced Serum Medium (Invitrogen) containing 10% FBS. In the 2nd transfection, either 200 nM siRNA for lamin, or 20 nM siRNA for GW182 were mixed with 40 nM siRNA for lamin A/C and then were transfected into HeLa cells using 7µL of Oligofectamine. In each single transfection mix was added to the well and the cells were incubated at 37°C in a CO2 incubator for 24 hrs, after which the media was replaced with normal growth media. After certain hours of growth the cells were fixed and processed for indirect immunofluorescent studies.

**Fluorescence Microscopy**

Indirect immunofluorescence analyses used adherent HeLa cells grown on glass coverslips in 6-well culture dishes. All transfected cells were rinsed first with phosphate buffered saline (PBS), fixed in 3% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.5% Triton X-100 at room temperature for 5 minutes. For colocalization studies, cells were incubated at room temperature for 1 hour with primary antibodies to the following proteins: GW182 (human serum, 1:6000), Ago2 (human serum, 1:200), lamin A/C (mouse monoclonal antibody, 1:100), LSm4 (rabbit polyclonal antibody, 1:200). After washing with PBS, cells were incubated with corresponding secondary fluorochrome-conjugated goat antibodies at room temperature for 1 hour. Alexa Fluor 488 (1:400), Alexa Fluor 568 (1:400) (Invitrogen) and Cy5
(1:100) (Jackson ImmunoResearch Laboratories) were the fluorochromes used. Lastly, the coverslips were mounted onto glass slides using VECTASHIELD Hard Set Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI, VECTOR Laboratories). Fluorescence images were obtained using a Zeiss Axiovert 200M microscope. Images of fixed cells were taken using 20x 0.75 NA or 40x 1.4 NA objectives.

Western Blotting

HeLa cells were grown on a 6-well plate without coverslip. In each experiment, transfected cells were harvested in RIPA buffer (150 mM NaCl, 1% TX-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.5) containing Complete protease cocktail inhibitors (Roche). Afterward the protein concentration of each sample was measured, equal amounts of protein extract were separated on a 10% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose membrane was blocked in 5% nonfat dried milk in PBS-Tween 1 hour at room temperature, then probed with primary antibody in appropriate dilution for 1 hour, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-human IgG (1:5000, Caltag) for 1 hour at room temperature. Immunoreactive bands were detected by the SuperSignal Chemiluminescent system (Pierce) according to the manufacturer’s instructions.

Statistical Analysis

The expression of lamin A/C in each cell was monitored individually based on the light intensity using the AxioVs40 software (Ver. 4.4.0.0, Carl Zeiss Vision GmbH). Images from a complete experiment were taken using the same exposure time and at least 100 cells were randomly selected for the measurement of lamin A/C intensity. Results were analyzed for statistical significance using Prism 4.0c for Macintosh (Graphpad Software Inc., San Diego,
The median values of lamin A/C signal in untransfected HeLa cells and lamin A/C-siRNA transfected HeLa cells were defined as 0 and 100% siRNA function respectively.

**Results**

**GW182 Is Essential for GWB Formation and Recruitment of Ago2 to GWB**

Although the integrity of GWB was shown important for efficient silencing activity, issue is raised against the unknown mechanism on how the dominant interfering constructs disrupted GWB (Jakymiw *et al.*, 2005). One may argue that it was the constructs themselves rather than disruption of GWB that interfered with the RISC activities. A different method to disassemble GWB is necessary to confirm the important role of GWB on RNAi. Here we employ a siRNA pool which contains 4 different individual siRNA targeting different sites along GW182 mRNA, to silence the expression of GW182 and lead to disassembly of GWB. To test the efficiency of siRNA pool silencing target GW182, HeLa cells were transfected with 200 nM of siRNA pool for 48, 72 and 96 hours and changes of GWB were monitored by staining with human anti-GW182 antibody. As shown, the majority of GWB were disassembled upon transfection of the siRNA pool for GW182 after 48 hours (Fig. 2-1). More complete disruption of GWB was achieved after 72 and 96 hours (Fig. 2-1). Furthermore, the localization of Ago2 and LSm4 to these cytoplasmic foci was completely abolished upon GW182-knockdown (Fig. 2-2). In summary, our data support that GW182 is essential for GWB formation and localization of Ago2 to GWB.

**Silencing of Lamin A/C Was Impaired upon Knockdown of GW182 and Disassembly of GWB Using a Co-transfection Strategy**

To test our hypothesis that GW182 and/or GWB are important for RNAi function, HeLa cells were transfected with siRNA specific for GW182, siRNA for lamin A/C, or both for 3 days, and double-stained with anti-GW182 and anti-lamin A/C antibody (Fig. 2-3A). GW182-siRNA
caused disassembly of GWB without significantly affecting the expression of lamin A/C. In contrast, both number and size of GWB increased in siRNA transfected cells where the expression of lamin A/C was efficiently silenced by siRNA specific for lamin A/C (Fig. 2-3A). Notably, co-transfection of siRNA specific for GW182 and siRNA specific for lamin A/C did not result in larger and greater number of GWB formation. Instead, it led to complete disassembly of GWB and the efficiency of lamin A/C-knockdown by siRNA was dramatically impaired (Fig. 2-3A). Western blot analysis of lamin A/C expression also demonstrated knockdown of lamin A/C was impaired in cells co-transfected with siRNA for GW182 and siRNA for lamin A/C, compared to that in cells transfected with siRNA for lamin A/C alone (Fig. 2-3B). Altogether, it is implicated that knockdown of GW182 abolishes the recruitment ability of siRNA and prevents formation of cytoplasmic foci which leads to inhibition of efficient RNAi activity. A potential issue of co-transfection of siRNA for GW182 and siRNA for lamin A/C is that the siRNA for GW182 may compete with that for lamin A/C on the intracellular RNAi machinery resulting in incomplete knockdown of either GW182 or lamin A/C. Although there was no evidence that the knockdown of GW182 or disruption of GWB was less complete in the cells transfected with mixed siRNA compared to those transfected with siRNA for lamin A/C alone, it is possible that the reduction of lamin A/C silencing in the mixing siRNA transfection may not be directly related to GWB disassembly but was a result from competitive usage of RNAi machinery.

Silencing of Lamin A/C Was Impaired by Sequential Transfection of GW182-siRNA and Lamin A/C-siRNA to HeLa Cells

To further confirm that GW182 and/or GWB are important for RNAi, we employed an alternative strategy using sequential transfection. HeLa cells were first transfected with either 200 nM siRNA targeting GW182 or 200 nM siRNA for luciferase and then two days later the
cells were transfected with 200 nM siRNA specific to lamin A/C (Fig. 2-4A). Consistent with
the co-transfection data above, GW182-knockdown significantly impaired RNAi activity while
transfection of luciferase siRNA did not (Fig. 2-4A). Quantification of lamin A/C expression
level in 100 cells for each setting demonstrated a 91% reduction of RNAi activity in GW182-
knockdown cells (Fig. 2-4B), which was statistically significant from that in control cells
transfected with siRNA for luciferase (Fig. 2-4B, P<0.001, Kruskal-Wallis test) but was not
significantly different from that in untransfected cells. In contrast, the reduction of RNAi
function was only 16% in cells transfected with luciferase siRNA which was not statistically
significant compared to that in cells with lamin A/C-siRNA alone (P>0.05). An alternative
consideration was that a substantially lower concentration of siRNA could be employed for these
analyses because the siRNA titration experiment showed that as low as 40 nM siRNA were
efficient in ~95% knockdown 48 hours post-transfection (data not shown). Thus experiments 2
and 3 as shown were performed with 40 nM of siRNA for GW182 or siRNA for luciferase in the
first transfection, which was followed by a second transfection of 40 nM siRNA for lamin A/C
48 hours later (Fig. 2-4B). Additional 20 nM siRNA for GW182 was included in the second
transfection with an attempt to maintain a lower level of GW182. In control cells, 20 nM siRNA
for luciferase was also added in the second transfection (Fig. 2-4B). Notably, reduction of RNAi
function in cells transfected with 200 nM of siRNA for GW182 (experiment 1) was more
dramatic than that in the cells with 40 nM of siRNA (experiment 2 and 3). Immunofluorescence
data were consistent with this result showing that disruption of GWB was more complete by
using 200 nM of siRNA for GW182 than 40 nM of siRNA upon 48-hour transfection (data not
shown), indicating reduction of RNAi activity may correlate with the degree of disassembly of
GWB. Again, our data demonstrates that GW182 and formation of GWB are important for efficient RNAi activity.

**Discussion**

In the present study, knockdown of GW182 by siRNA using both co-transfection and sequential transfection strategy resulted in disruption of GWB, abolishment of the localization of Ago2 to GWB, and inhibition in siRNA-mediated silencing. Our data is consistent with our recent observation that disruption of GWB using independent dominant negative constructs of GW182 or Ago2 led to impaired siRNA silencing activity (Jakymiw *et al.*, 2005). The implication that RNA silencing is localized to these cytoplasmic foci is intriguing and open interesting potentials for improving RNAi in experimental manipulation and potential therapeutic applications.

**Increased Number and Size of GWB upon siRNA Transfection May Correlate with mRNA Degradation Targeted by RISC**

In this report, we observed that transfecting lamin A/C-siRNA induced larger and greater numbers of GWB where GW182 and Ago2 localize. The number and size of GWB reached a peak on the 3rd day post-transfection (data not shown). The fact that detectable changes of GWB take place at 24 hours after the initial silencing activity of the transfected siRNA suggests the changes are related to mRNA degradation targeted by RISC, which is consistent with the proposed function of GWB in mRNA decay. Moreover, the number and size of GWB may correlate with the amount of mRNA need to be degraded within the cytoplasm. It is supported by that cells transfected with siRNA for luciferase, a protein doesn’t exist in human cells, did not have increased GWB. This may be due to no RISC-targeted mRNA sitting on GWB and no recruitment of decay factors for mRNA degradation. Nevertheless, it is possible that GWB may also involve in helping RISC to target and cleave mRNA at earlier stage of RNAi activity.
Chances are earlier changes of GWB were too minor to detect. Overall, our results suggest siRNA is capable of localizing target mRNA to GWB and forming larger foci to participate potentially in silencing activity. This model is supported by recent studies which indicate that miRNA is very important for recruiting the targeted mRNA to GWB (Liu et al., 2005b).

**Integrity of GWB May Be Required for Efficient RNAi Function**

In our laboratory, three different strategies have been used to disassemble GWB and examine the effect of GWB on RNAi: 1. Dominant negative effect using two different constructs (the C-terminal half of Ago2 and the N-terminal 1/3 of GW182) (Jakymiw et al., 2005); 2. Co-transfection strategy in which siRNA for GW182 and lamin A/C were co-transfected into cells; 3. Sequential strategy in which siRNA for GW182 was transfected into cells prior to a second transfection of siRNA for lamin A/C. All these strategies have consistently demonstrated impaired RNAi function upon GWB disassembly. In contrast, cells with intact GWB showed efficient silencing activity of siRNA on its target. The limitation of present study is that RNAi mechanism was used to silence GW182 which is obvious very important for RNAi silencing process, leading to self-limiting compromised knockdown of GW182. This incomplete knockdown of GW182 may conceal the importance of GW182 on RNAi, although GW182-knockdown by 200 nM siRNA in our study already greatly affected the siRNA mediated-silencing.

From the results altogether, we propose that GW182 help to form GWB by recruiting Ago2/RISC, siRNA/miRNA, targeted mRNA, mRNA decay factors, and other proteins that are required for RNAi or mRNA decay. GWB provide a platform or microenvironment where Ago2, siRNA/miRNA, and targeted mRNA can be positioned properly and where targeted mRNAs are cleaved by RISC with high efficiency. Although evidence has been shown that purified Ago2 with siRNA can manage to cleave the targeted mRNA *in vitro*, the localization of RISC to GWB
may greatly increase the cleavage efficiency \textit{in vivo}. It is supported by the data that abolishing localization of Argonaut 2/RISC to GWB by either overexpression of dominant-negative constructs or GW182-knockdown greatly inhibited RISC activity. Moreover, GWB may also help to recycle siRNAs thereby increasing the knockdown efficiency of the target. However, further studies will be needed to elucidate these mechanisms.

Figure 2-1. SMARTpool siRNA for GW182 caused the disassembly of GWB. HeLa cells transfected with siRNA for GW182 for 48, 72, and 96 hours, were stained with index human anti-GW182 antibody (green). Nuclei were counterstained with DAPI (blue). Compared with untransfected cells in panel i showing many cells with GWB (arrows), majority of GWB were disassembled in cells transfected with siRNA for GW182 after 48 h (ii). More complete disruption of GWB was achieved after 72 h (iii) and 96 h (iv). Few cells (u) with apparently intact GWB were observed representing potentially untransfected cells. Bar, 10 μm.
Figure 2-2. GW182-knockdown by siRNA completely disassembled GWB and abolished the localization of Ago2 and LSm4 to the foci. HeLa cells were transfected with 200 nM pooled siRNA for GW182 for 72 hours, stained with index human anti-Ago2 serum (green, ii) and rabbit anti-LSm4 (red, iv). Nuclei were counterstained with DAPI (blue). Compared with untransfected cells stained with anti-Ago2 (i) and anti-LSm4 (iii) showing many cells with cytoplasmic foci that Ago2 and LSm4 enriched in (arrows), the localization of Ago2 and LSm4 to these cytoplasmic foci was completely abolished in cells transfected with siRNA specific for GW182 after 72 h (ii, iv). In addition to localization to GWB, LSm4 is also known to be involved in RNA splicing function in the nucleus (iii, iv) which is not affected by the GW182-siRNA transfection. Merge images are shown in the bottom row. Bar, 10 μm
Figure 2-3. The lamin A/C-knockdown by siRNA was impaired upon disassembly of GWB by co-transfecting lamin A/C-siRNA with GW182-siRNA. A) HeLa cells were transfected with 100 nM siRNA for GW182 (iii, iv), 100 nM siRNA for lamin A/C (v, vi), or both (vii, viii) for 3 days, and were processed for double IIF with human anti-GW182 antibody (green) and mouse monoclonal antibody to lamin A/C (magenta). Nuclei were counterstained with DAPI (blue). GW182-siRNA caused disassembly of GWB (iii) as expected without affecting the expression of lamin A/C (iv, compared to ii). In contrast, the transfection of siRNA for lamin A/C showed both increased in number and size of GWB (v, arrows) and efficient silencing of lamin A/C (vi). Notably, co-transfection of siRNA for GW182 and lamin A/C resulted in almost complete disassembly of GWB (vii, compared to i and iii) and dramatically impaired...
lamin A/C expression (viii, compared to vi). Arrows, GWB. Bar, 10 μm. B) Western blot analysis of lamin A/C expression demonstrating inhibition of siRNA silencing activity by co-transfection of siRNA specific for GW182. Tubulin levels were monitored to confirm the equal loading of samples.
Figure 2-4. RNAi function was greatly affected by sequential transfection of GW182-siRNA and lamin A/C-siRNA. A) Transfection of siRNA for GW182 disassembled GWB and significantly inhibited RNAi. Cells were co-stained with index human anti-GW182 antibody (green) and mouse monoclonal anti-lamin A/C antibody (magenta). Nuclei were counterstained with DAPI (blue). The expression of lamin A/C was efficiently silenced either in cells singly transfected with 200 nM siRNA to lamin A/C (iv) or in sequential transfection experiments where 200 nM control siRNA for luciferase was transfected prior to the addition of 200 nM siRNA for lamin A/C (viii). Note that both conditions obviously increased the size and number of GWB to different degrees (iii, vii, compared to i and v). In contrast, cells first transfected with siRNA for GW182 showed disassembly of GWB (v) and impaired silencing of lamin A/C in the 2nd transfection by siRNA for lamin A/C (vi). Arrowheads show examples of cells.
with disrupted GWB and failure to silence lamin A/C. Some cells (*) with significant silencing of lamin A/C have a few detectable GWB (v, arrows). Arrow, GWB. Bar, 10 μm. B) Quantification of the effect of GW182 knockdown and GWB disassembly on siRNA functional activity. Results of three experiments using different concentrations of siRNA are presented. The lamin A/C intensities for at least 100 cell nuclei were measured for each data point and the value of untransfected and lamin A/C-siRNA transfected cell nuclei were established as 0 and 100% siRNA function. The percent siRNA function was calculated based on median value of lamin A/C intensity in each group. Cells transfected with siRNA for GW182 had significant decrease in RNA silencing function (9–31%) compared to those transfected with control luciferase (luc) siRNA (P<0.001, Kruskal-Wallis test). In contrast, cells transfected with siRNA for luciferase yielded between 76% to 93% siRNA function and were not statistically different from control with transfection of lamin A/C-siRNA alone (P>0.05, **). In experiment 1 using highest concentration of siRNA (200 nM) for each transfection, intensity of lamin A/C in GW182-siRNA transfected group did not show statistically difference with that of the untransfected group (P>0.05, *).
CHAPTER 3
SMALL INTERFERING RNA-MEDIATED SILENCING INDUCES TARGET-DEPENDENT ASSEMBLY OF GW/P BODIES

Introduction

GW bodies (GWB), also known as mammalian processing bodies (P bodies), are cytoplasmic foci that contain multiple decay factors and are involved in the 5'→3' mRNA degradation pathway. GWB are named from the marker protein GW182, which contains multiple glycine (G) and tryptophan (W) repeats and a classic RNA binding domain at the carboxyl terminal (Eystathioy et al., 2002a). The mRNA decay factors/complexes found in GWB include the deadenylase Ccr4, the decapping complexes Dcp1a/1b/Dcp2, the LSm1-7 complex, Ge-1 (also known as Hedls), rck/p54, and exonuclease Xrn1 (Bashkirov et al., 1997; van Dijk et al., 2002; Ingelfinger et al., 2002; Lykke-Andersen, 2002; Eystathioy et al., 2003b; Cougot et al., 2004; Andrei et al., 2005; Yu et al., 2005; Fenger-Gron et al., 2005). GWB are physically juxtaposed to and transiently interact with stress granules (SG), which process cytoplasmic aggregates of stalled translational preinitiation complexes that accumulate during stress responses and share certain components with GWB (Kedersha et al., 2005).

In addition to mRNA decay, a crucial role of GWB and their components in RNA interference (RNAi) was recently uncovered (Anderson and Kedersha, 2006; Jakymiw et al., 2007; Eulalio et al., 2007a). RNAi is a post-transcriptional gene silencing mechanism that uses specific double-stranded RNA to silence genes in a sequence-specific manner (Mello and Conte, Jr., 2004; Meister and Tuschi, 2004). In brief, the double-stranded RNA is processed by Dicer into small interfering RNA (siRNA) or microRNA (miRNA). The 21–26 nucleotide siRNA and miRNA are then incorporated in the effector complex, RNA-Induced Silencing Complex (RISC), which either cleaves or inhibits translation of the target mRNA. In 2005, two key components of RISC, Argonaute2 (Ago2) and siRNA/miRNA, were found to be enriched in GWB (Sen and
MiRNA-targeted mRNA also localizes to GWB in a miRNA-dependent manner (Liu et al., 2005b). These observations provide the first evidence that RNAi is linked to GWB and opened a new era in our understanding of intracellular RNAi processing. In addition, Ago2 interacts with GW182 in human cells (Jakymiw et al., 2005; Liu et al., 2005a) and this interaction is conserved in *C. elegans* and *Drosophila* (Ding et al., 2005; Behm-Ansmant et al., 2006). Disruption of GWB either by a dominant negative effect or by GW182-knockdown impairs siRNA and miRNA activities (Jakymiw et al., 2005; Liu et al., 2005a), indicating that GW182 and/or GWB are important for RNAi function. Furthermore, miRNA-mediated mRNA degradation requires GWB components such as GW182, the decapping complex Dcp1/Dcp2 and the deadenylase Ccr4:Not (Rehwinkel et al., 2005; Behm-Ansmant et al., 2006), whereas miRNA-mediated translational repression requires rck/p54 (Chu and Rana, 2006).

GWB are highly dynamic structures. First, GWB change in size and number in response to cell proliferation, nutrient conditions and the cell cycle. GWB are larger and more numerous in proliferating cells whereas they are apparently fewer in resting and nutrient starved cells (Yang et al., 2004). During cell cycle, smaller GWB are seen in early S phase and larger GWB are seen in late S and G2 phases. The majority of GWB disassembled prior to mitosis and small GWB reassembled in early G1 (Yang et al., 2004). Second, as sites for the 5’ → 3’ mRNA decay, the size and number of GWB are affected by blocking deadenylation, decapping, 5’→3’ mRNA degradation or translation (Sheth and Parker, 2003; Cougot et al., 2004; Andrei et al., 2005; Teixeira et al., 2005). GWB require RNA for assembly and the amount of mRNA or mRNA decay intermediates accumulated in GWB affects the size and number of these foci (Sen and Blau, 2005; Brengues et al., 2005; Teixeira et al., 2005). Third, and more interestingly, our
recent studies show that blocking the genesis of miRNA disassembles GWB and introducing siRNA into these cells re-assembles these foci, implicating that either miRNA or the miRNA activities are crucial for the formation of GWB (Pauley et al., 2006). Since siRNA is very similar to miRNA structurally, we were interested to determine whether siRNA or siRNA-mediated activities also have an effect on the assembly of GWB. The answer to this question will help us understand the correlation between RNAi activity and the formation of GWB.

**Materials and Methods**

**Antibodies**

The human prototype anti-GWB (anti-GW182 and anti-Ago2) sera were obtained from serum banks at the Advanced Diagnostics Laboratory, University of Calgary. The selection of sera was based on specific reactivity to either GW182 or Ago2 (Jakymiw et al., 2006). Rabbit anti-Ago2 was a gift from Dr. Tom Hobman (University of Alberta, Edmonton, Canada) and rabbit anti-Dcp1a was obtained from Dr. Jens Lykke-Andersen (University of Colorado). Rabbit polyclonal anti-LSm4 was produced as described previously (Eystathioy et al., 2002b). Mouse monoclonal anti-lamin A/C 636, anti-TIAR and anti-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), BD Biosciences (San Jose, CA), and Sigma-Aldrich (St. Louis, MO), respectively. Rabbit polyclonal anti-GFP and anti-rck/p54 were purchased from Invitrogen Corporation (Carlsbad, CA) and MBL International Corporation (Boston, MA), respectively. Chicken polyclonal anti-LSm1 was purchased from GenWay Biotech Incorporated (San Diego, CA).

**Small Interfering RNA**

The siRNAs used in the current study were all purchased from Dharmacon (Lafayette, CO). The siRNAs were dissolved in 1x Universal buffer (provided by Dharmacon) and the resulting 20μM stock was stored in aliquots at -20°C prior to use. The pre-designed siRNAs
include siCONTROL siRNA for human/mouse/rat lamin A/C (cat# D-001050-01-05), siCONTROL RISC-Free siRNA (cat# D-001220-01) and siGENOME SMARTpool siRNA for human RAGE (cat# M-003625-01). The sense and antisense strand of the rest of siRNAs with known sequence are listed below respectively: Individual siGENOME ON-TARGET Human TNRC6 (GW182) siRNA duplex: 5’-GAA AUG CUC UGG UCC AUU-3’ and 5’-P UAG CGG ACC AGA GCA UUU CUU-3’ (cat# D-014107-01-0020); hAgo2: 5’-GCA CGG AAG UCC AUC UGA A dTdT-3’ and 5’-UUC AGA UGG ACU UCC GUG C dTdT-3’ (Chu and Rana, 2006); hrck/p54: 5’-GCA GAA ACC CUA UGA GAU UUU-3’ and 5’-AAU CUC AUA GGG UUU CUG CUU-3’ (Chu and Rana, 2006); hLSm1: 5’-GUG ACA UCC UGG CCA CCU CAC UU-3’ and 5’-GUG AGG UGG CCA GGA UGU CAC UU-3’ (Chu and Rana, 2006); hLamin A/C: 5’P-CUG GAC UUC CAG AAG AAC A dTdT-3’ and 5’-Cy3-UGU UCU UCU GGA AGU CCA G dTdT-3’; Luciferase GL2 duplex: 5’-CGU ACG CGG AAU ACU UCG A dTdT-3’ and 5’-U CGA AGU AUU CCG CGU ACG dTdT-3’ (cat# D-001100-01-20); EGFP: 5’-P GGC UAC GUC CAG GAG CGC ACC-3’ and 5’-P U GCG CUC CUG GAC GUA GCC UU-3’

**Construction of Inducible GFP3T3 Fibroblast (TRE-GFP3T3) Cells**

To establish a reliable 3T3 fibroblast cell line expressing tTA, both constructs (pCAG 20–1 and pUHD10-3 Puro) (Era and Witte, 2000) were transfected into 3T3 cells by Fugene 6 (Roche, Indianapolis, IN) and selected with 1 µg/ml puromycin in doxycycline-free medium. Clones, which proliferated in doxycycline-free medium but died in the presence of doxycycline (1 µg/ml) (Sigma, St Louis, MO) and puromycin (1 µg/ml) were selected as primary parental doxycycline-regulatory 3T3 cells. The open reading frame of EGFP was amplified by polymerase chain reaction (PCR) using LA-Taq polymerase (TAKARA Bio, Otsu, Japan) from pCX-GFP vector (Ikawa et al., 1995). Primers used were; 5’- TGCCGACGCGTGCCACC
ATGGTGAGCAAGG, and 5′-ATAAGAATGCGGCCGCTGAGGAGTGAATTCTTACTT.
The PCR fragment was ligated into the MluI-NotI restriction site of the pTRE2hyg expression vector (Clontech, Palo Alto, CA), which contained a tetracycline-responsive element. This vector was introduced into the doxycycline-regulated 3T3 cells by Fugene 6 and selected with hygromycin B (200 µg/ml) (Invitrogen, Carlsbad, CA). Doxycycline-dependent expression of EGFP was confirmed by the GFP expression with or without doxycycline (1 µg/ml).

**Cell Culture and Transfection**

HeLa, HSG, NIH 3T3 and GFP3T3 cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO₂. SiRNA was transiently transfected into cells grown on glass coverslips in a six-well plate using Oligofectamine (Invitrogen). Briefly, the cultured cells were grown to 30-40% confluency. Then 100 nM or, in the case of co-transfection of two different siRNAs, 100 nM of each siRNA was transfected into cells. Usually, cells were fixed 2 days after the transfection. In the 4-day time point experiment, cells were fixed at day 1, day 2, day 3 and day 4 after transfection. In the sequential transfection experiment, the second siRNA transfection was performed 24 hours after the initial transfection and the cells were fixed at 2 or 3 days after the second transfection. In the plasmid and siRNA co-transfecting experiment, HeLa cells were grown to 50-70%. Then the GFP vector was co-transfected with siRNA either for Ago2, or for rck/p54, or for LSm1 at 1:3 ratio (w/w) using Lipofectamine 2000 (Invitrogen). To test the efficiency of Ago2-knockdown by siRNA, GFP-Ago2 was co-transfected with siRNA for Ago2 at 1:1 ratio (w/w) and then the cells were lysed 2 days later. The transfected cells from all the above experiments were either processed for indirect immunofluorescence (IIF) or lysed for western blot analysis.
**Western Blot Analysis**

Cells were harvested in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, PH 7.5) containing complete protease cocktail inhibitors (Roche). When whole cells lysate was used to detect the expression level of GWB components upon siRNA transfection, cells were lysed in Laemmli sample buffer directly. Afterwards, equal amounts of protein extract were separated on 7.5% or 10% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose membrane was blocked in 5% non-fat dried milk in PBS-Tween for 1 hour at room temperature, and then probed with primary antibodies to the following proteins for 1 hour: Ago2 (1:200), Dcp1a (1:1000), rck/p54 (1:500), LSm1 (1:2000), LSm4 (1:200), Tubulin (1:3000) and GFP (1:200). The membrane was then incubated with horseradish-peroxidase-conjugated goat antibodies for 1 hour and immunoreactive bands were detected by the Supersignal Chemiluminescent system (Pierce).

**Fluorescence Microscopy**

Cells were fixed and permeabilized as previously described (Jakymiw *et al.*, 2005). For colocalization studies, cells were incubated at room temperature with primary antibodies to the following proteins for 1 hour: GW182 (human serum, 1:6000), lamin A/C (1:100), Dcp1a (1:500), rck/p54 (1:500) and TIAR (1:100). Afterwards, cells were incubated with the corresponding secondary fluorochrome-conjugated goat antibodies at room temperature for 1 hour. Alexa Fluor 488 (1:400), Alexa Fluor 568 (1:400), Alexa Fluor 350 (1:100) (Invitrogen) and Cy5 (1:100) (Jackson ImmunoResearch Laboratories, West Grove, PA) were the fluorochromes used. Last, glass coverslips were mounted onto the glass slides using either Vectashield Mounting Medium with or without 4’,6-diamidino-2-phenylindole (DAPI, VECTOR Laboratories). Fluorescent images were captured with a Zeiss Axiovert 200M microscope fitted with a Zeiss AxioCam MRm camera using x10 0.75NA, x20 0.75NA, x40 0.75 NA or x63 1.4
NA objectives. All the exposure times and gain settings within one set of experiment are equivalent. Color images were processed using Adobe Photoshop (San Jose, CA) version 7.

Statistical Analysis

GWB/P bodies in each cell were monitored based on light intensity using the Axio Vs40 software (Ver. 4.5.0.0, Zeiss). Images from a complete experiment were taken using the same exposure time and about 2 to 3 different areas (100–300 cells) were randomly selected for the measurement of the number of GWB using CellProfiler object counting software program (Carpenter et al., 2006). The threshold was set to a value so that the background signal was erased and the quantitated foci were confirmed by being overlaid with the original image. The number of foci in each cell was counted by correlating the position of each focus with the area around each nucleus which was defined as the coverage of a cell. Statistical analysis was performed using Prism 4.0c for Macintosh (Graphpad Software Inc., San Diego, CA). Data between groups were compared using Kruskal-Wallis with Dunn’s multiple comparison tests or Fisher’s exact test with Bonferroni’s correction. For the measurement of RNAi activity, about 70 to 110 cells from each data group were randomly selected for the measurement of lamin A/C intensity using the AxioVs40 software. The area from each cell nuclei was selected based on DAPI staining and then switched to lamin A/C staining for measurement. The median values of lamin A/C signal in the mock transfected (or luciferase siRNA-transfected) HeLa cells and in the luciferase siRNA and lamin siRNA sequentially transfected (or lamin siRNA singly transfected) HeLa cells were defined as 0 and 100% siRNA function, respectively. In the sequential transfection experiment, the lamin A/C silencing efficiency of the sample group was calculated based on \[
\frac{\text{median fluorescent intensity in mock group} - \text{median fluorescent intensity in sample group}}{\text{median fluorescent intensity in mock group} - \text{median fluorescent intensity in luciferase siRNA and lamin A/C-siRNA sequentially transfected group}} \times 100%.
\]
In the co-transfection
experiment the lamin A/C silencing efficiency was calculated based on similar formula: (median fluorescent intensity in luciferase siRNA group – median fluorescent intensity in sample group) / (median fluorescent intensity in luciferase siRNA group – median fluorescent intensity in lamin A/C-siRNA group) x 100%.

Results

The Size and Number of GWB Increased in Cells Transfected with siRNA Eliciting RNA Silencing of Its Endogenous Target

To address the question of how siRNA affects the formation of GWB, we transfected HeLa cells with lamin A/C-siRNA, which targets an endogenous mRNA, or luciferase siRNA, which does not target an endogenous mRNA. Interestingly, we detected larger and greater numbers of GWB in cells with efficient lamin A/C-knockdown by siRNA than in the mock transfected cells or in the cells transfected with luciferase siRNA (Fig. 3-1AB). The accumulation of rck/p54 in GWB also increased in lamin A/C-siRNA-transfected cells (Fig. 3-1A). In comparison, cells transfected with luciferase siRNA had comparable GWB to those in the mock transfected cells (Fig. 3-1A). In addition, another siRNA for a different endogenous target, RAGE (Receptor for Advanced Glycation End-product) (Bierhaus et al., 2005), also induced larger and greater numbers of GWB (Fig. 3-2). Notably, the “RISC-free” siRNA, a siRNA chemically modified by Dharmacon to lose its silencing ability, is similar to luciferase siRNA in that it did not affect the size or number of GWB either (Fig. 3-1D). Taken together, these data suggested that siRNA which elicited RNA silencing of its endogenous target was able to increase the size and number of GWB. Interestingly, the protein expression level of GWB components, including Ago2, Dcp1a, rck/p54 and LSm4, did not increase upon transfection of siRNA for lamin A/C (Fig. 3-1C). This supports a hypothesis that these components were recruited to GWB from preexisting or nascent pools of protein upon the transfection of siRNA for lamin A/C.
In addition to HeLa cells, we transfected siRNA for lamin A/C into a different cell line, human salivary gland cell line (HSG), and a similar increase of GWB was detected (Fig. 3-1E). This demonstrated that the observed effect of siRNA on GWB was not restricted to HeLa cells. As will be shown in subsequent experiments, this siRNA-induced increase of GWB is also observed in mouse cells.

To exclude the possibility that the transfected siRNA might act like certain stressor, such as sodium arsenite, which is reported to induce the formation of both GWB and SG (Kedersha et al., 2005), we examined the effect of lamin A/C-siRNA on the formation of SG (Fig. 3-3). As a result, only numerous large GWB but no anti-TIAR (a marker protein for SG) labeled SG was detected in lamin A/C-siRNA-transfected cells (Fig. 3-3). In comparison, many GWB and SG were observed in the arsenite-treated cells, a positive control for the stress response, where GWB and SG are often juxtaposed (Fig. 3-3). This data indicated that the siRNA-induced increase of GWB was independent of stress response.

Small Interfering RNA Required Endogenous Expression of Its Target for Inducing an Increase in Size and Number of GWB

To further verify that the siRNA-induced increase of GWB is dependent on the presence of the siRNA target, we transfected siRNA for GFP into a mouse fibroblast cell line (NIH 3T3) engineered to express GFP (GFP3T3) integrally (Fig. 3-4). Mock transfected GFP3T3 cells or NIH 3T3 cells transfected with GFP-siRNA served as controls that either missed the siRNA or the target of siRNA, respectively (Fig. 3-4). Our data showed that the siRNA for GFP efficiently silenced the expression of its target (Fig. 3-4A, 3-5) and induced a prominent increase of GWB only in the GFP3T3 cells (Fig. 3-4A). By using the CellProfiler object counting software (Carpenter et al., 2006) to quantitate GWB in each cell, we showed that the number of GWB per cell in GFP-siRNA-transfected GFP3T3 cells was significantly higher than that either in the
mock GFP3T3 cells or in the GFP-siRNA-transfected NIH 3T3 cells (Fig. 3-4B). In addition, the percentage of cells with GWB or with increased GWB in the GFP-siRNA-transfected GFP3T3 cells was also remarkably higher than those in the mock GFP3T3 cells and in the GFP-siRNA-transfected NIH 3T3 cells (Fig. 3-4C). In comparison, there was no significant difference between the mock transfected NIH 3T3 cells and GFP-siRNA-transfected NIH 3T3 in either the number of GWB per cell or the percentage of cells with GWB (or with increased GWB) (Fig. 3-4BC). These data demonstrated that GFP-siRNA induced an increase in both the number of GWB and the percentage of cells with GWB only in the 3T3 cells expressing GFP, a finding supporting that the siRNA-induced increase of GWB is target-dependent and may correlate with siRNA-elicited silencing activities.

**The siRNA-Induced Increase of GWB Started on Day 1 after Transfection and Lasted for at Least 4 Days**

To determine the temporal increase of GWB induced by siRNA, we performed a 4-day time point experiment and monitored the changes of GWB as well as the accumulation of Dcp1a in GWB at each time point. Interestingly, after transfection of siRNA for lamin A/C, GWB were much larger on day 3 and 4 than on day 1 (Fig. 3-6A, 3-7A). Quantitative analysis showed that the average number of GWB in lamin A/C-siRNA-transfected cells was higher than that of the mock cells or luciferase siRNA-transfected cells through day 1 to 4. The maximal increase of GWB was approximately 5-fold of mock cells and occurred on day 3 (Fig. 3-6B, 3-7B). In addition, the percentage of cells with GWB in lamin A/C-siRNA-transfected cells (88%–98%) was higher than that of the other two groups (35%–67%) through day 2 to 4 (Fig. 3-6C). In comparison, the number of GWB through day 1 to day 4 was similar in luciferase siRNA-transfected cells and the mock transfected cells (Fig. 3-6B). The percentage of cells with GWB was also similar between these two groups (Fig. 3-6C). Notably, the variation of GWB (in size
and number) during cell cycle was more easily detected in these two groups than in the lamin A/C-siRNA-transfected cells (Fig. 3-7 and data not shown). A similar temporal increase in the accumulation of rck/p54 in GWB was observed (Fig. 3-6A and data not shown). Taken together, these data demonstrated that siRNA that elicits mRNA silencing could induce an increase both in the number of GWB and in the percentage of cells with GWB. This increase in number and size of GWB occurred on day 1 after transfection and lasted for at least 4 days.

**GW182 Was Required for the siRNA-Induced Increase of GWB**

GW182 is important for both GWB formation (Yang et al., 2004) and miRNA/siRNA activity (Jakymiw et al., 2005; Liu et al., 2005a; Chu and Rana, 2006). We were interested to examine how the knockdown of GW182 affects the siRNA-induced increase of GWB. We used the same GW182-siRNA tested previously to be efficient in silencing the target (Jakymiw et al., 2005). As shown, the transfected GW182-siRNA disassembled GWB and abolished the accumulation of Dcp1a in foci without affecting the level of lamin A/C expression (Fig. 3-8A). Quantitative analysis confirmed that GW182-siRNA-transfected cells only had 0.22 fold of the GWB in mock cells (Fig. 3-8B). More interestingly, numerous large GWB induced by lamin A/C-siRNA were absent in cells where siRNA for GW182 and lamin A/C were co-transfected and where RNA silencing was impaired (Fig. 3-8A). This observation was supported by the quantitative data which indicated the number of siRNA-induced GWB dropped from 3.11 fold to 0.25 fold upon GW182 knockdown (Fig. 3-8B). Luciferase siRNA served as a control siRNA that did not affect the assembly of GWB. In summary, these data indicated that the siRNA-induced increase of GWB required GW182, suggesting that the integrity of GWB and/or RNAi activity are very important for the siRNA-induced increase of GWB.
The siRNA-Induced Increase of GWB Required Ago2 and Correlated with RNA Silencing Activities

To further determine the correlation between RNAi activity and the siRNA-induced increase of GWB, we were interested to knockdown Ago2, another GWB component that is a key component of RNA silencing (Liu et al., 2004). The siRNA used in this study was shown to efficiently silence the expression of Ago2 by both another group and us (Fig. 3-9A) (Chu and Rana, 2006). Interestingly, when co-transfecting the Ago2 siRNA and the GFP vector at a 3:1 ratio (w/w), we detected discrete foci in the GFP positive cells, which likely contained the siRNA for Ago2 (Fig. 3-9B). Consistently, the number of GWB (labeled by anti-Dcp1a) in Ago2-knockdown cells were only slightly less than control cells (0.75 fold, Fig. 3-9CE), indicating Ago2 is not essential for the formation of GWB. To examine the effect of Ago2-knockdown on the siRNA-induced increase of GWB, Ago2 siRNA and lamin A/C-siRNA were co-transfected into HeLa cells. As a control, luciferase siRNA was co-transfected with lamin A/C-siRNA. Notably, Ago2-knockdown impaired the silencing function of lamin A/C-siRNA (60% remained, Fig. 3-9D) and abolished the increased size and number of GWB (labeled by anti-Dcp1a) induced by lamin A/C-siRNA, resulting in the number of foci decreasing from 1.77 fold to 0.81 fold (Fig. 3-9CE). Ago2-knockdown also decreased the percentage of cells with foci induced by lamin A/C-siRNA, which dropped to a percentage similar to that of control cells (Fig. 3-9F). In comparison, cells transfected with siRNA for lamin A/C and luciferase had high efficiency of RNA silencing (97.8%) and large numbers of prominent foci (Fig. 3-9CD). Taken together, these data indicated that Ago2 was not essential for GWB formation but was required for the siRNA-induced increase of GWB. The observation that knockdown of Ago2 inhibited the siRNA-induced increase of GWB strongly suggested that impairment of RNAi function affects GWB dynamics.
Knockdown of LSm1 or Rck/p54 Did not Inhibit the Assembly of GWB Induced by siRNA

To further dissect the correlation of RNAi activity with the siRNA-induced increase of GWB, we performed knockdown experiments for LSm1 and rck/p54, both reported to be important for the formation of GWB but have no effect on siRNA-mediated silencing (Chu and Rana, 2006). The siRNA for LSm1 and rck/p54 are shown by us (Fig. 3-10A) and other investigators (Chu and Rana, 2006) to inhibit the expression of its target. To confirm the roles of the LSm1 and rck/p54 in the formation of GWB, we co-transfected a GFP vector with siRNA either for LSm1 or for rck/p54 at a ratio of 1:3 (w/w) into HeLa cells for 2 days (Fig. 3-10B). GFP vector was co-transfected with luciferase siRNA as a control. GWB were barely detected in rck/p54-knockdown cells (Fig. 3-10B). In comparison, a few small GWB were observed in LSm1-knockdown cells (Fig. 3-10B), implying that LSm1 may be required for the formation of a subset of GWB. In general, both LSm1-knockdown and rck/p54-knockdown prevented the formation of large prominent GWB induced by lamin A/C-siRNA (Fig. 3-10C and data not shown). Nevertheless, sequentially transfecting lamin A/C-siRNA reassembled many small GWB in rck/p54-knockdown cells, resulting in the percentage of cells with GWB increasing drastically from 7% (0.07 GWB/per cell) to 86% (~20 GWB/per cell) (Fig. 3-10C and data not shown). Apparently, the residual rck/p54 was recruited to the newly assembled GWB in rck/p54-knockdown cells, despite the highly efficient silencing of rck/p54 in these cells (Fig. 3-10C, 3-11). In LSm1-knockdown cells, sequentially transfecting lamin A/C-siRNA reassembled fewer small GWB (Fig. 3-10C). Furthermore, Cy3 labeled lamin A/C-siRNA localized to these reassembled GWB and efficiently silenced the expression of its target both in LSm1-knockdown cells and in rck/p54-knockdown cells (Fig. 3-10C). Notably, the localization of Cy3-lamin A/C siRNA to GWB was not only found in Cy3-siRNA strongly transfected cells, but also found in Cy3-siRNA weakly transfected cells which was more easily detected in rck/p54-knockdown
cells (Fig. 3-10C). Consistent with the above data and data from others (Chu and Rana, 2006),
knockdown of LSm1 or rck/p54 did not affect siRNA-mediated silencing (Fig. 3-10D). Most
importantly, the assembly of these siRNA-induced GWB correlated with the silencing of lamin
A/C both in LSm1-knockdown cells (87%) and in rck/p54-knockdown cells (79%) (Fig. 3-10E).
In summary, LSm1 and rck/p54 contributed to the formation of GWB to different degrees.
However, knockdown of either did not prevent the siRNA-induced assembly of GWB or
localization of siRNA to GWB. The siRNA-induced assembly of GWB in LSm1-knockdown
cells and in rck/p54-knockdown cells correlated with RNA silencing activities.

Discussion

The major and highly reproducible observation reported in the current study is that
siRNA:mRNA induces the appearance of numerous large GWB in the majority of cells where
the normal variation of GWB in size and number during the cell cycle was greatly obscured,
whereas in untransfected cells large GWB were only detected in a small fraction of cells at late S
and G2 stage of the cell cycle. Further study indicated that this siRNA-induced increase in size
and number of GWB was regulated by RNAi activity. Our results provide novel insights into the
correlations between siRNA function and the assembly of GWB, suggesting that GWB could
serve as markers for siRNA-mediated activity in mammalian cells.

Small Interfering RNA:mRNA Initiates Assembly of Microscopic Detectable GWB by
Recruiting GWB Components

siRNA:mRNA nucleated the assembly of GWB possibly by recruiting GWB components.
We hypothesize that GWB components are actively exchanged between the cytoplasmic pool
and GWB based on the actively ongoing siRNA/miRNA function and the mediated mRNA
decay/translational repression. One model is that siRNA:mRNA is targeted to or recruits
components to pre-existing submicroscopic GWB, which then develop into larger cytoplasmic
structures detectable by conventional microscopy. Alternatively, siRNA:mRNA itself forms de novo GWB by recruiting necessary components/complexes for silencing.

The greatest numbers of large GWB were induced on day 3 after siRNA transfection suggesting that the large GWB may be more related to siRNA-mediated mRNA decay processes. We postulate that the smaller GWB (or the submicroscopic GWB), which increased on day 1 or even earlier, may be related to the early stage of RNA silencing. The formation of large GWB could be attributed to the siRNA-mediated degradation of large amounts of mRNAs, which may have exceeded the maximal capacity of the mRNA decay machinery. As recently proposed, this could then lead to accumulation of these mRNAs or mRNA decay intermediates in GWB (Franks and Lykke-Andersen, 2007). Similarly, increased accumulation of mRNA decay intermediates in GWB due to possible interference by Cy3 dye in the degradation of target mRNA is a reasonable interpretation for why Cy3-lamin A/C siRNA induces more numerous large GWB than does unlabeled lamin A/C-siRNA with exactly same sequence (data not shown).

The Role of GWB Components for the Assembly of GWB

Based on the requirement of different GWB components examined in this study for the assembly of GWB, we can deduce some scenarios for GWB assembly. Since GW182 and rck/p54 are important for miRNA-mediated decay/translational repression, their requirement in GWB formation may be attributed, at least in part, to the amount of miRNA-mediated repressed mRNPs maintained in GWB. Notably, GW182-knockdown greatly inhibited the reassembly of GWB induced by siRNA:mRNA resulting in very few detectable GWB. This may suggest that GW182 is required at the early stage of GWB assembly. In contrast, rck/p54-knockdown prevented the formation of large GWB but not small GWB induced by siRNA:mRNA suggesting that rck/p54 may function at a later stage after the initial trigger of GWB formation. Moreover, rck/p54-knockdown may limit the amount of mRNPs shuttled to one GWB and the excess RNPs
have to be shuttled to other “unsaturated” GWB, thereby forming more numerous but smaller
detectable GWB. Furthermore, Cy3-lamin A/C siRNA localized to these reassembled small
GWB and mediated efficient silencing indicating that small GWB are capable of carrying out
RNA silencing. It is possible that the RNA decay in small GWB is less efficient than that in
large GWB; however, this speculation will need to be addressed in future experiments.
Interestingly, even in cells with efficient rck/p54-knockdown the residual rck/p54 was detected
and concentrated in the reassembled GWB (Fig. 3-11) suggesting that the recruitment of rck/p54
to GWB is very efficient. This recruitment may be via siRNA:mRNA-associated Ago2 since
rck/p54 directly interacts with Ago2 (Chu and Rana, 2006). It is possible that rck/p54
contributes to the assembly of these newly formed GWB or it is recruited there for downstream
function. Nevertheless, we cannot differentiate whether the assembly of these siRNA-induced
GWB is required for siRNA-mediated silencing or is the consequence of siRNA-mediated
silencing. Since almost complete knockdown of rck/p54 barely affected RNA silencing
efficiency, we postulate that, in general, rck/p54 is not important for siRNA function, unless it
efficiently fulfills functions with only residual amounts of the protein.

In contrast to GW182 and rck/p54, LSm1 has a less profound effect on the assembly of
GWB. The incomplete disassembly of GWB by LSm1-knockdown was previously reported
(Andrei et al., 2005) whereas complete disappearance of GWB by LSm1-knockdown was
reported in another study (Chu and Rana, 2006). The reasons for this discrepancy can be
attributed to different ways of defining foci, different ways of determining cells with
knockdown, or different efficiency/specificity of the antibody used to detect foci. Nevertheless,
our data are in agreement with the conclusion that LSm1 contributes to the formation of GWB.
The reassembly of GWB induced by siRNA:mRNA in LSm1-knockdown cells implies that
LSm1, like rck/p54, is not required to initiate the assembly of GWB and is possibly involved in the formation of larger foci.

Similar to LSm1, Ago2 has less profound effect on the assembly of GWB compared to GW182 and rck/p54. Knockdown of Ago2 had a minor effect on the accumulation of Dcp1a indicating that Ago2 is not required to stabilize mRNA decay factors in GWB and that the mRNA processing stage could be independent of the siRNA/miRNA-mediated silencing stage. Furthermore, the function of Ago2 in miRNA-mediated translational repression could possibly be compensated by other Argonaute proteins in mammalian cells. This explanation was supported by previous studies where Ago2-knockdown did not affect miRNA function profoundly (Chu and Rana, 2006) and where Agos1-4 had equal capability in binding miRNAs (Meister et al., 2004).

**Regulation of GWB Assembly**

An understanding of the regulation of GWB assembly in mammalian cells has been greatly advanced by the current study. Our data suggest that, in mammalian cells, the majority of mRNAs degraded via the 5’→3’ pathway or translationally repressed in GWB are mediated by siRNA or miRNA. We speculate that under certain circumstances GWB may serve as markers for siRNA/miRNA activity and, therefore, the variation in number and size of GWB may correlate with the activities of miRNA during different stages of the cell cycle and proliferation (Yang et al., 2004; He et al., 2005; O'Donnell et al., 2005; Hatfield et al., 2005; Lian et al., 2006). Interestingly, a recent publication reported that *Drosophila* siRNA:mRNA or miRNA:mRNA also nucleated the formation of GWB (Eulalio et al., 2007b), an observation that strongly supports our finding that siRNA/miRNA-mediated function is a key regulatory mechanism of GWB assembly. Nonetheless, their data also implicated differences in the regulation of GWB
formation in *Drosophila* from that in human. For example, Ago2-knockdown disassembled GWB and long dsRNA did not restore GWB in LSm1-knockdown or rck/p54-knockdown cells in *Drosophila* (Eulalio *et al*., 2007b). These apparent discrepancies with our data may be attributed to the potential difference in the function of GWB components and in the RNAi pathway between *Drosophila* and human cells (Okamura *et al*., 2004; Lee *et al*., 2004).

Depending on the presence of the RNAi machinery, the regulation of GWB assembly might vary between species. For example, the RNAi machinery as well as related cofactors, such as Argonaute proteins and GW182, are absent in *S. cerevisiae*. The absence of RNAi in *S. cerevisiae* may explain the observed differences between yeast P bodies and mammalian P bodies (GWB) in responding to stresses (Sheth and Parker, 2003; Yang *et al*., 2004; Brengues *et al*., 2005; Teixeira *et al*., 2005). As yeast P bodies are considered sites for processing global messages, GWB are more like specific cellular structures regulating and organizing siRNA/miRNA-mediated function. This is consistent with the concept that most mRNAs are degraded via the 5’ → 3’ pathway in yeast, whereas in mammalian cells only a portion of mRNAs are degraded via the 5’ → 3’ pathway while the majority of mRNAs are degraded via the 3’ → 5’ by exosome-mediated process (Wilusz *et al*., 2001; Tourriere *et al*., 2002; Coller and Parker, 2004; Parker and Song, 2004). The correlations between GWB and RNAi are proven to be strong.

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Figure 3-1. Transfection of siRNA for lamin A/C increased the size and number of GWB. A) Lamin A/C-siRNA induced an increase in the size and number of GWB (ii compared to i, v compared to iv) in HeLa cells whereas luciferase siRNA did not (iii compared to i, vi compared to iv). HeLa cells were mock transfected or transfected with siRNA for either lamin A/C or luciferase. Cells were fixed 3 days after transfection and stained with index human anti-GWB serum (green, i-iii) or rabbit anti-rck/p54 (green, iv-vi) for visualizing GWB and mouse anti-lamin A/C to monitor the knockdown of lamin A/C (blue, i-vi). Scale bar, 10μm. B) Western blot analysis demonstrated that siRNA for lamin A/C achieved efficient gene silencing. The level of tubulin...
reactivity served as a loading control. C) Expression of components of GWB was not apparently affected upon transfection of siRNA either for lamin A/C or luciferase. HeLa cells were transfected with 100 nM siRNA either for lamin A/C or luciferase and lysed 3 days later. The whole cell lysates were analyzed by western blot for the expression of GWB components including Ago2, Dcp1a, rck/p54, and LSm4. The level of tubulin reactivity served as a loading control. D) Transfection of RISC-free siRNA had no detectable effect on GWB (green, iv compared to i) as luciferase siRNA (green, iii compared to i). HeLa cells were mock transfected (i, v) or transfected with 100 nM lamin A/C-siRNA (ii, vi), or luciferase siRNA (iii, vii), or RISC-free siRNA (iv, viii). Cells were fixed on day 3 after transfection and stained with human anti-GWB serum for GWB (green), mouse anti-lamin A/C for detecting the knockdown of lamin A/C (magenta) and DAPI for nuclei (blue). The average number of GWB per cell is shown with the total number of cells counted indicated in parentheses. Scale bar, 10 μm. E) Transfection of lamin A/C-siRNA induced more GWB (iii–iv compared to i–ii, ix–x compared to vii–viii) in human salivary gland (HSG) cell line on both day 3 and 4 after transfection whereas luciferase siRNA did not (v–vi compared to i–ii, xi–xii compared to vii–viii). The HSG cells were mock transfected or transfected with 100 nM siRNA either for lamin A/C or luciferase. The transfected cells were fixed 3 and 4 days after transfection and then stained with human anti-GWB serum (magenta) and rabbit anti-Dcp1a (green) for GWB, mouse anti-lamin A/C (blue) for detecting the knockdown of lamin A/C. The average number of GWB per cell is shown with the total number of cells counted indicated in parentheses. Scale bar, 10 μm.
Figure 3-2. Transfection of siRNA for RAGE activated assembly of GWB. RAGE-siRNA was transfected into HeLa cells to examine its effects on GWB formation. Mock transfected cells or cells transfected with siRNA for luciferase served as controls. Cells were fixed on day 3 after transfection and stained with human anti-GWB serum (green) and counterstained with DAPI (blue). The number and size of GWB increased only in cells transfected with siRNA for RAGE (ii). Scale bar, 10μm.
Figure 3-3. Lamin A/C-siRNA did not induce stress granules. Lamin A/C-siRNA induced numerous large GWB (viii compared to ii) but did not induce SG (vii compared to i and iv). In contrast, sodium arsenite induced SG (iv compared to i) and an increase of GWB (v compared to ii). An enlarged cell section is shown in the bottom right corner for arsenite-treated cells (iv-vi), illustrating that SG were often adjacent to GWB (inset of vi). The cells were counterstained with human anti-GWB (magenta), mouse anti-TIAR for SG (green) and DAPI for nuclei (blue). Merged image of each row are shown in the right column. Scale bar, 10μm.
Figure 3-4. The siRNA-induced increase of GWB is target-dependent. A) GFP-siRNA induced an increase in the size and number of GWB in GFP3T3 cells (iv compared to i) but did not in NIH 3T3 cells (vii compared to i). Expression of GFP is efficiently inhibited in GFP-siRNA-transfected GFP3T3 cells (green, v compared to ii). The cells were fixed on day 3 after mock transfection or transfection of GFP-siRNA, and were co-stained with human anti-GWB serum (magenta, i, iv, vii) for GWB and DAPI for nuclei (blue). Merged image of each row is shown in the right column. Scale bar, 10μm. B-C) Quantitative analyses indicated that GFP-siRNA induced an increase both in the number of GWB per cell and in the percentage of cells with GWB in GFP3T3 cells. The number of GWB per cell was counted in 100 to 200 cells for each group. Each dot represented a single cell and was plotted on a graph with the number of GWB per cell as Y-axis (B). The median with the interquartile range is indicated for each data group. *, significant difference between groups indicated by bracket (Dunn’s multiple comparison test, P<0.001); ns, no significant difference between groups indicated by bracket (Dunn’s multiple comparison test, P>0.05).
Based on the data from (B), the percentage of cells with GWB (open bars) or increased GWB (filled bars) was calculated and shown for each individual group (C). Seven foci per cell, the median value of GFP-siRNA-transfected GFP3T3 group, are set as the standard value to define cells with increased GWB. The bars indicated by * are significantly higher than the others (Fisher’s exact test, P<0.001).
Figure 3-5. Green fluorescence protein (GFP) was efficiently knocked down by siRNA. GFP3T3 cells were either mock transfected (iii, iv) or transfected with 100 nM siRNA for GFP (i, ii), and then fixed 3 days later. Nuclei were counterstained with DAPI (blue). Scale bar, 10μm.
Figure 3-6. The increases of GWB started on day 1 and were most prominent on day 3 after transfection of siRNA for lamin A/C. A) HeLa cells were mock transfected, or transfected with 100 nM siRNA for lamin A/C or for luciferase, and then fixed on day 1 (i-vi), day 2 (vii-xii), day 3 (xiii-xviii) and day 4 (xix-xxiv) after transfection. Cells were counterstained with human anti-GWB serum (magenta) and rabbit anti-Dcp1a (green) to monitor the changes of GWB. The level of lamin A/C was evaluated by using mouse anti-lamin A/C (blue). Scale bar, 10μm. B) Quantitative analysis indicated that cells transfected with lamin A/C-siRNA had a significant increase in the average number of GWB per cell through day 1 to day 4. The number of GWB per cell was quantitated as described in Fig. 3-4 and Methods. The groups indicated by * are significantly higher than the other groups on the same day (Dunn’s multiple comparison test, P<0.001). C) The percentage of cells with GWB in lamin A/C-siRNA-transfected cells (filled square) is significantly higher than mock cells (filled circle) and luciferase siRNA-transfected cells (filled triangle) through day 2 to 4.
(Fisher exact test, P<0.001). In comparison, the other two groups were very similar to each other (Fisher exact test, no significant difference from day 1 to 3, P>0.05).
Figure 3-7. The increase of GWB induced by lamin A/C-siRNA was prominent on day 3. HeLa cells were either mock transfected (i) or transfected with 100 nM siRNA for lamin A/C (ii) or luciferase (iii), and then fixed on day 3 after transfection. Cells were counterstained with index human anti-GWB serum (green) to monitor GWB and DAPI for nuclei (blue). A) A majority of the cells transfected with siRNA for lamin A/C had larger and greater numbers of foci. Scale bar, 10 μm. B) Quantitative analysis of the number of foci per cell showed that both the percentage of cells with foci and the average number of foci per cell increased in cell treated with lamin A/C-siRNA. The number of foci per cells was quantitated as described in Fig. 3-4B and methods. The median with the interquartile range is indicated for each data group. The lamin A/C-siRNA group, as indicated by bracket, was significantly higher than the other two groups (Dunn’s multiple comparison test, P<0.001); ns, no significance (Dunn’s multiple comparison test, P>0.05).
Figure 3-8. GW182 was required for the siRNA-induced increase of GWB. A) GW182-knockdown inhibited both the increase of GWB (x-xi compared to xiii-xiv) and the lamin A/C-knockdown (xii, compared to xv) induced by lamin A/C-siRNA. HeLa cells were transfected with 100 nM siRNA for GW182 (iv-vi), 100 nM siRNA for lamin A/C (vii-ix), or both (x-xii) for 3 days. SiRNA for luciferase in both the single siRNA transfection (i-iii) and co-transfection (xiii-xv) served as controls. Cells were stained with human anti-GWB serum (magenta) and rabbit anti-Dcp1a (green) for GWB, mouse anti-lamin A/C for monitoring lamin A/C-knockdown (blue). Scale bar, 10µm. B) Quantitative analysis showed that knockdown of GW182 abolished the siRNA-induced increase of GWB. The number of GWB in ~300 cells collected from 3 randomly selected fields was counted for each group. The average number of GWB per cell in each group was divided by that of mock cells to calculate the fold difference. The bars indicated by * are significantly higher than others (Fisher’s exact test, P<0.001).
Figure 3-9. The siRNA-induced increase of GWB required Ago2 and correlated with RNA silencing activities. A) Western blot analysis showed that siRNA for Ago2 efficiently knocked down Ago2. GFP-Ago2 was co-transfected with siRNA either for Ago2 or for lamin A/C at 1:1 ratio (w/w) for 2 days. Tubulin reactivity served as a loading control. B) Ago2-knockdown barely affected the formation of GWB. SiRNA for Ago2 (i-ii) or luciferase (iii-iv) was co-transfected with GFP vector (green) at 3:1 ratio (w/w) into HeLa cells for 2 days. The cells were counterstained with rabbit anti-Dcp1a (red). In cells transfected with Ago2 siRNA, discrete GWB were detected in GFP-positive cells (i-ii, arrows), which was comparable to the GWB in GFP negative cells in the same panel (i-ii, arrowhead) or to the GWB in GFP-positive cells transfected with luciferase siRNA (iii-iv, arrows). Scale bar, 10 μm. C-F) SiRNA-induced increase of GWB was abolished when Ago2 was knocked down and RNA silencing efficiency was impaired. HeLa cells were transfected with 100 nM siRNA for Ago2 (i, ii), 100 nM siRNA for lamin A/C, or both (v, vi) for 2 days. SiRNA for luciferase in both the single siRNA transfection and co-transfection (iii, iv) served as controls. Cells were stained with human anti-GWB serum (red) and rabbit anti-
Dcp1a (green) for GWB, DAPI for nuclei (blue), mouse anti-lamin A/C for monitoring lamin A/C-knockdown (lamin A/C staining is not shown). C) Ago2 siRNA diminished the increase of GWB induced by lamin A/C-siRNA. Fewer GWB were observed in cells co-transfected with siRNA for Ago2 and lamin A/C (v, vi) than cells with siRNA for both luciferase and lamin A/C (iii, iv). Scale bar, 10μm. D) Quantitative analysis of lamin A/C silencing efficiency indicated that Ago2-knockdown impaired RNAi activity. AxioV40 software was used to measure the fluorescent intensity of nuclear lamin A/C staining in each cell for each group (70 cells per group). The median value of lamin A/C fluorescent intensity in each group was used to calculate its corresponding RNA silencing efficiency based on the formula described in Methods. Y axis, lamin A/C silencing efficiency. E) Quantitative analysis showed that Ago2-knockdown significantly decreased the average number of GWB induced by siRNA. The number of GWB in each cell was counted as described in Fig. 3-4B and Methods. Then the resulted average number of GWB per cell in each group was divided by that of control cells to calculate the fold difference. The bars indicated by * are significantly higher than others (Fisher’s exact test, P<0.001). F) Ago2-knockdown decreased the percentage of cells with GWB induced by siRNA. Based on the data from (E), the percentage of cells with GWB was calculated and shown for each individual group. The bars indicated by * are significantly higher than other groups (Fisher’s exact test, P<0.001).
Figure 3-10. Knockdown of LSm1 or rck/p54 disassembled GWB but did not inhibit the assembly of GWB induced by siRNA. A) Knockdown of LSm1 or rck/p54 in HeLa cells by siRNA. HeLa cells were transfected with siRNA for LSm1 or rck/p54, harvested 2 days later and analyzed by western blot with antibodies to LSm1 or rck/p54. Tubulin reactivity served as a loading control. B) Rck/p54 is required for the assembly of majority of GWB whereas LSm1 is important only for a portion of GWB. GFP vector were co-transfected with siRNA for LSm1, rck/p54, or luciferase at 1:3 ratio (w/w) into HeLa cells for 2 days. The transfected cells were counterstained with
human anti-GWB serum (magenta) and rabbit anti-rck/p54 (green). Merge images of anti-GWB and anti-rck/p54 are shown in the right column. The average number of GWB per cell and the percentage of cells with GWB are shown with the total number of cells counted indicated in parentheses. Scale bar, 10μm. C) Lamin A/C-siRNA induced the assembly of GWB and localized to these GWB in spite of LSm1-knockdown or rck/p54-knockdown. 100 nM siRNA either for LSm1 (iv-vi) or rck/p54 (vii-xii) were transfected into HeLa cells and 24 hours later 100 nM Cy3 labeled lamin A/C-siRNA (red) were sequentially transfected. The transfected cells were fixed 3 days after the 2nd transfection and stained with human anti-GWB serum (green), mouse anti-lamin A/C (magenta) and DAPI (blue). Merged images of Cy3-siRNA and anti-GWB are shown in the right column. The percentages of cells exhibiting a similar or identical GWB staining to the cells presented are shown in panel iv and x with the total number of cells counted indicated in parentheses. Panels iv-ix show representative localization of siRNA to GWB in Cy3-siRNA strongly transfected cells whereas panels x-xii show the localization in weakly transfected cells. Insets are enlarged by 1.5 to 2 folds and the Cy3 signal is enhanced to show the localization of siRNA to GWB. Arrows indicate the co-location for the weak Cy3 signals in enlarged insets. Arrowheads indicate the co-localization for the strong Cy3 signals in GWB. Scale bar, 10μm. D) Knockdown of LSm1 or rck/p54 did not affect RNA silencing activities. AxioV40 software was used to measure the fluorescent intensity of nuclear lamin A/C staining in each cell for each group (~100 cells per group). The median value of lamin A/C fluorescent intensity in each group was used to calculate its corresponding RNA silencing efficiency based on the formula described in Methods. Y axis, lamin A/C silencing efficiency. E) The siRNA-induced assembly of GWB correlated with RNA silencing activities. One hundred to 150 cells from each group are randomly selected and subjected to quantitation according to the assembly of GWB and the knockdown of lamin A/C. GWB +, cells exhibiting similar or identical GWB staining to the cells presented in panel C, iv for LSm1-knockdown or panel C, x for rck/p54-knockdown; GWB -, cells without microscopic detectable GWB; Lamin KD +, the fluorescent intensity of nuclear lamin A/C staining is lower than 50% of the median value of lamin A/C fluorescent intensity of the mock cells; Lamin KD -, the lamin A/C intensity is higher than 50% of the median value of lamin A/C intensity of mock cells.
Figure 3-11. Lamin A/C-siRNA induced assembly of GWB and recruited residual rck/p54 to the reassembled GWB in rck/p54-knockdown cells. HeLa cells were either mock transfected (i-iii) or transfected with 100 nM siRNA for rck/p54 (iv-vi) for 3 days. In the sequential transfection, 100 nM lamin A/C-siRNA were transfected 24 hours after the initial transfection of siRNA for rck/p54 and the cells were fixed 2 days after that 2\textsuperscript{nd} transfection (vii-ix). The transfected cells were stained with human anti-GWB serum (green), rabbit anti-rck/p54 (red), mouse anti-lamin A/C (magenta) and DAPI (blue). Merge image of anti-rck/p54 and anti-GWB are shown in the right column. Insets are enlarged about 1.5-fold and the rck/p54 signal in rck/p54-knockdown cells (vii, ix) is enhanced to show the localization of rck/p54 to GWB. Arrowheads indicate co-localization. Scale bar, 10\textmu m.
CHAPTER 4

THE C-TERMINAL HALF OF AGO2 BINDS TO MULTIPLE GW-RICH REGIONS OF GW182 AND REQUIRES GW182 TO MEDIATE SILENCING

Introduction

MicroRNA (miRNA)-mediated gene silencing is an important post-transcriptional regulation which controls in part the half lives of mRNA targets. In this regulation, miRNA binds to the 3’-UTR of mRNA leading to translational inhibition, mRNA degradation, and mRNA sequestration (Nilsen, 2007). MiRNAs are evolutionarily conserved in most of species and are estimated to regulate ~30% of protein-encoding genes in human (Lewis et al., 2005; Filipowicz et al., 2008).

There are four human Ago proteins that include Ago1 to Ago4, which are the core components of silencing effector complexes and are known to bind single-stranded miRNA. These Ago proteins share greater than 80% identity and are primarily characterized by PAZ and PIWI domains. The PAZ domain contains a binding pocket for the 3’ overhanging nucleotides of miRNA. Interestingly, despite highly conserved sequences, only the PIWI domain of Ago2 harbors RNase H-type activity and, therefore, Ago2 also functions in siRNA-mediated slicing of mRNA targets (Liu et al., 2004; Yuan et al., 2005). Tethering Ago proteins to the 3’-UTR of mRNA mimicked miRNA function and effected translational repression (Pillai et al., 2004).

GW182 is important for miRNA-mediated translational silencing and interacts with Ago2. GW182 contains several glycine/tryptophan-rich (GW-rich) regions, a glutamine/asparagine-rich (Q/N-rich) domain, and a C-terminal RNA recognition motif (RRM) (Eystathioy et al., 2002a; Decker et al., 2007). GW182 localized to and was essential for the formation of GW bodies (GWB, also known as mammalian P bodies) (Yang et al., 2004; Schneider et al., 2006), cytoplasmic structures closely linked to mRNA decay (Sheth and Parker, 2003; Eystathioy et al., 2003b) and the miRNA/siRNA pathway (Jakymiw et al., 2005; Pauley et al., 2006; Lian et al.,
2006; Lian et al., 2007). Knockdown of GW182 greatly impaired miRNA-mediated gene silencing and subsequent mRNA degradation (Rehwinkel et al., 2005; Liu et al., 2005a).

Interestingly, Ago proteins, miRNAs, and mRNAs targeted by miRNAs all colocalized with GW182 in GWB (Sen and Blau, 2005; Pillai et al., 2005; Jakymiw et al., 2005; Liu et al., 2005b). Furthermore, GW182 interacted with Ago2 and this interaction was conserved from plants to human (Ding et al., 2005; Jakymiw et al., 2005; Liu et al., 2005a; Behm-Ansmant et al., 2006; El-Shami et al., 2007; Till et al., 2007). However, the role of GW182 and the importance of GW182-Ago2 interaction in translational repression remain unclear. In the current study, we mapped the GW182-Ago2 interaction and investigated the possible role of this interaction in miRNA-mediated silencing in human.

**Materials and Methods**

**Construction of Deletion Constructs of GW182 and Ago2**

The details of constructing GW1Δ1 (aa254–751, formerly known as GW182Δ1), Ago2 (aa1–860) and PIWI (aa478–860) were described previously (Jakymiw et al., 2005). To construct pENTR-TNR (aa1–204), PCR amplification was conducted on the human testis cDNA (BioChain) using primer 5’-TTT GGA AGA TCT ATG AGA GAA TTG GAA GCT AAA GCT-3’ and primer 5’-AAG GGA AGT GCC ATT CAT ACC-3’, which is downstream of an internal Kpn I site (nt1252). The 1.5kb PCR product was digested with BglII and KpnI to generate a 1.2kb fragment that was used to replace the 5’ 500bp BamHI-KpnI fragment of pENTR-GW182. Afterwards, the pENTR-GW182 with the extra N-terminal ~750nt was digested with BamHI (nt610) and NotI (3’ end linker) to release a 6.5kb fragment comprising a majority of GW182. The vector fragment containing the N-terminal region was ligated at room temperature (RT) for 1 hour to generate pENTR-TNR (aa1–204). GW1Δ1a (aa254–503) was
constructed by PCR using primer 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAA CGC CAT GGA TGC TGA TTC T-3’, and primer 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG GGA AGT GCC ATT CAT ACC TG-3’. Annealing temperature was 55.7°C for first 2 cycles and then 62.5°C for additional 30 cycles. GW1Δ1b (aa502 – 751) was constructed by PCR using primer 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAC TTC CCT TTC TCA CCT TAG CA-3’, and primer 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG GCC TCT GTC CCA TTG TCA GT-3’. Annealing temperature was 55.2°C for the first 2 cycles and then 62.4°C for additional 30 cycles. GW1Δ7 (aa1034 – 1962) was constructed by PCR using primer 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAA AGA CCA GCA AGC ACA GGT ACA-3’, and primer 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA GAC CTT GTA CAA GAA AGC TGG GTT AGG CAA CAT CAA GGC ATA G-3’. Annealing temperature was 55.7°C for the first 2 cycles and 63.3°C for additional 30 cycles. The human Ago3 mutant (Ago3m) in pCMV-SPORT6 vector was obtained from Invitrogen (Clone number: CS0DB008YP10). Ago3m sequence was amplified by PCR using primer 5’-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GGA AAT CGG CTC CGC AGG ACC C-3’, and primer 5’-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA CAC TTT GTC CCA TTG TCA GT-3’. Sequences, GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CA and GGG GAC CAC TTT GTA CAA GAA AGC TGG GT, common in the above forward and reverse primers were designed to incorporate the recombination sites. The products from the above PCR reactions were then cloned into pDONR207 (Invitrogen) using the Gateway BP recombination reaction as per the manufacturer’s instructions (Invitrogen). To construct pENTR-GW1Δ5 (aa1670 – 1962), pENTR-GW182 was digested with SalI (5’ end linker) and SpeI (nt5008) to release a 4.2Kb vector fragment containing the C-terminus of GW182. The overhangs of this
fragment were filled in and then ligated. To construct pENTR-MGW (aa566 – 1343), phrGFP-KIAA1460 was digested with \textit{XhoI} (5’ end linker) and \textit{SmaI} to release a 2.3Kb fragment, which was then subcloned into the \textit{SalI} and \textit{EcoRV} sits of pENTR2B (Invitrogen). To construct pENTR-PAZ (aa1 – 480), pENTR-Ago2 was digested with \textit{XhoI} (nt1467) and \textit{XhoI} (3’ end linker) to generate the vector fragment containing N-terminal half of Ago2, which was purified and then ligated. To construct pENTR-Ago1, EST clone pBluescript hAgo1 was first digested with \textit{BamHI} (3’ end linker) and the overhang was filled in to generate a blunt 3’ end. Then the digested product was cut by \textit{KpnI} (5’ end linker) to generate a 4.0 kb fragment which was subcloned into the \textit{KpnI} and \textit{EcoRV} sites of pENTR1A (Invitrogen). To construct pENTR-Ago4, EST clone pBluescript hAgo4 was digested with \textit{SmaI} (5’ end linker) and \textit{ScaI} (3’ end linker) to generate a 3.5 Kb fragment which was then subcloned into the \textit{DraI} and \textit{EcoRV} sites of pENTR1A (Invitrogen). All of the variants used in current study were subcloned into Gateway compatible GST, GFP or 3xFlag vectors by using Gateway LR recombination reaction (Invitrogen). pIreSneo-Flag/HA Ago3 was obtained from Thomas Tuschi (Meister \textit{et al.}, 2004) through Addgene. The tethering assay plasmids including pClneo-NHA vector, NHA-Ago2, Renilla luciferase RL-5BoxB and FL were gifts from Dr. Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Switzerland (Pillai \textit{et al.}, 2004). To generate NHA-PIWI (aa478–860) and NHA-PAZ (aa1–480), pClneo-NHA vector was converted to gateway destination vector using the Gateway Vector Conversion System (Invitrogen). Then PIWI (aa478–860) and PAZ (aa1–480) were moved from corresponding pENTR vectors to the pClneo-NHA gateway vector respectively by recombination. All DNA constructs used in this study were confirmed by direct DNA sequencing.
Antibodies

Rabbit anti-Ago2 and rabbit anti-GST were gifts from Dr. Tom Hobman (University of Alberta, Edmonton, Canada) and Dr. Peter Sayeski (University of Florida, Gainesville, USA), respectively. Mouse monoclonal anti-HA was purchased from Covance (Emeryville, CA). Mouse monoclonal anti-Flag M2 and anti-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-GFP was purchased from Invitrogen Corporation (Carlsbad, CA).

Plasmid Transfection, GST Pull-down, and Western Blot Analysis

HeLa cells were cultured in DMEM containing 10% fetal bovine serum in a 37°C incubator with 5% CO2. HeLa cells were grown to 90-100% in 6-well plate at the day of transfection. GST-tagged construct was singly transfected or co-transfected with other tagged constructs into HeLa cells using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions for 24 h. For detection of expression of GST-tagged proteins in whole cell lysate, the cells were lysed in Laemmli sample buffer directly. For GST pull-down assay, the cells were lysed with NET/NP40 buffer (150mM NaCl, 5mM EDTA, 50mM Tris, pH 7.4, 0.3% NP40) with Complete Protease Cocktail Inhibitor (Roche Diagnostics) and then sonicated at 20% amplitude for 10 sec for 3 times on ice. Afterwards, the lysates were centrifuged at 13,200 rpm for 5 min. The pellets (insoluble fractions) were lysed in Laemmli sample buffer directly. The soluble fractions were incubated with Glutathione Sepharose™ 4B (GE Healthcare) and mixed at 4°C for 2 h for GST pull-down. After the incubation, the beads were washed with NET/0.3% NP40 buffer for four times and the samples eluted in Laemmli sample buffer. The soluble fraction of cell lysates (input), GST pull-down samples, whole cell lysates, and insoluble fractions were separated on 10% polyacrylamide gel and transferred to nitrocellulose. Western blotting was performed as described previously (Lian et al., 2007). The dilutions of primary antibodies were:
1:1000 for anti-GST, 1:400 for anti-Flag, 1:1000 for anti-GFP, and 1:500 for anti-Ago2, 1:1000 for anti-HA.

**Indirect Immunofluorescence**

Cells were fixed and permeabilized as described previously (Jakymiw et al., 2005; Lian et al., 2007). The dilution of anti-Flag antibody was 1:1000.

**Tethering Assay Using a Dual Luciferase System**

HeLa cells were grown to about 90–100% confluence in 24-well plate at the day of transfection. To determine the effect of tethering PIWI (aa478 – 860) and PAZ (aa1 – 480), the cells were transfected with 0.1ng of constructs expressing reporter Renilla luciferase (RL-5BoxB), 100ng of control firefly luciferase (FL) plus 700ng of NHA tag, NHA-Ago2, NHA-PIWI, or NHA-PAZ using Lipofectamine 2000 (Invitrogen) for 48 h as per the manufacturer’s instructions. Cells were harvested 48 hour after transfection and the FL and RL activities were measured using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer’s protocol. Relative luciferase activities (ratio of targeted luciferase activities over control luciferase activities) were calculated as described previously (Pillai et al., 2004). Briefly, FL/RL activity in NHA vector transfected (control) group was regarded as 0% translational repression. The repression levels of other experimental groups were calculated by the percentage reduction of relative luciferase activities compared to that in NHA control group. The assay was performed in triplicates and repeated for 2 to 3 times. To detect the expressions of tethered NHA-tagged proteins, the above cell lysates were mixed at 1:1 ratio with Laemmlli sample buffer and western blot was performed as described in “Western Blot Analysis” of Materials and Methods.
RNA Interference and Quantitative Real Time PCR

The sequence of siRNA for GW182 or for GFP was described previously (Lian et al., 2007). To determine how GW182-knockdown affects the Ago2- or PIWI-mediated suppression, cells were grown to 30-50% confluence and were transfected with 100 nM of siRNA for GW182, or siRNA for GFP as a negative control using Lipofectamine 2000 (Invitrogen). Thirty hours later, these cells were transfected again with constructs expressing reporter RL-5BoxB, control FL and NHA tagged constructs as described above. Forty-eight hours after the second transfection, total RNA was extracted from HeLa cells using RNeasy Mini Kit (Qiagen, Valencia, CA). RNase-Free DNase Set (Qiagen) was applied to eliminate potential DNA contamination. The relative mRNA level of GW182 was measured in duplicate using ΔΔCt method (Livak and Schmittgen, 2001) and TaqMan® Fast Universal Master Mix (Applied Biosystems) with the corresponding TaqMan® Gene Expression Assay (TNRC6A, Hs00379422_m1, Applied Biosystems). The level of 18S rRNA was measured as internal control (18S rRNA, 4310893E, Applied Biosystems). The melting curve in each individual measurement was monitored to guard against non-specific amplification.

Results

C-terminal Half of Human Ago2 Containing the PIWI Domain Was Responsible for the Interaction with GW182

The interaction between human Ago2 and GW182 was first reported in 2005 (Jakymiw et al., 2005; Liu et al., 2005a). To further characterize this interaction, a series of deletion constructs covering different domains of GW182 and Ago2 were generated (Fig. 4-1A). The human GW182 gene identified as TNRC6A in the GenBank database is currently predicted to have 2 isoforms: GW182 as previously described (Eystathioy et al., 2002a) and a longer alternative-spliced product (NM_014494.2) that contains an extra N-terminal 253aa-polypeptide...
with a glutamine-repeat (Q-repeat) region encoded by CAG trinucleotide repeats (TNR). The deletion construct TNR (aa1 – 204) included the Q-repeat region and did not overlap with GW182 (Fig. 4-1A). TNR (aa1–204) and other two GW182 deletion constructs GW1Δ1 (aa254 – 751) and MGW (aa566 – 1343), respectively representing approximately the N-terminal 1/3 and middle 1/3 of GW182, were initially used to analyze the binding to Ago2 using the GST pull-down assay. GST-GW1Δ1 and GST-MGW were demonstrated to interact with endogenous Ago2 whereas GST-TNR did not (Fig. 4-1B). To map the region of Ago2 responsible for this interaction, two deletion constructs corresponding to the N-terminal (PAZ, aa1 – 480) and C-terminal (PIWI, aa478 – 860) halves were constructed. By co-expression of GST- and Flag-tagged constructs and the GST pull-down assay, Ago2 consistently interacted with GW1Δ1 and MGW but not with TNR (Fig. 4-1C). Importantly, PIWI but not PAZ was responsible for the association of Ago2 with GW1Δ1 and MGW (Fig. 4-1C). Notably, there was less of GST-MGW in the soluble input when it was co-transfected with Flag-Ago2 or Flag-PIWI than when GST-MGW was co-transfected with Flag-PAZ (Fig. 4-1C). To investigate the reason for these observed differences, the expression of GST-MGW was examined in both total cell lysates and the insoluble fractions. The expression of GST-MGW in total cell lysates was relatively uniform no matter which Flag-tagged constructs it co-transfected with (Fig. 4-2). Interestingly, higher levels of GST-MGW was observed in the insoluble fraction when it was co-transfected with Flag-Ago2 and even higher when co-transfected with Flag-PIWI, whereas GST-MGW was barely detectable when co-transfected with Flag-PAZ. This data suggested that MGW formed insoluble complexes with Ago2 or PIWI, which might explain why there was a lower level of MGW in the soluble input for the GST pull-down assays. In summary, GW182 fragments
GW1Δ1 and MGW both bound to Ago2 and this binding was mediated by the C-terminal half of Ago2.

**GW182-Ago2 Interaction Was Important for the Localization of Ago2 in Cytoplasmic Foci**

Previous studies have shown that Ago2 colocalized with GW182 in cytoplasmic GWB and GW182 was essential for the formation of these foci (Yang *et al.*, 2004; Jakymiw *et al.*, 2005). However, the driving force for the localization of Ago2 to GWB remains unknown. Based on the above data that the GW182 fragment MGW formed insoluble complexes with Ago2 and PIWI, but not with PAZ, we hypothesized that the GW182-Ago2 interaction is crucial for Ago2 to localize to GWB. To examine this hypothesis, Flag-Ago2, -PIWI or -PAZ was co-expressed with GFP-MGW in HeLa cells and Flag-PIWI or -PAZ were expressed alone as controls (Fig. 4-3). Flag-Ago2 was shown to colocalize with GFP-MGW in cytoplasmic foci whereas singly expressed Flag-PIWI or -PAZ were diffusely distributed in the cytoplasm (Fig. 4-3). Interestingly, co-expression of GFP-MGW with Flag-PIWI dramatically changed the distribution of Flag-PIWI, which was recruited to cytoplasmic foci and colocalized with GFP-MGW (Fig. 4-3). In contrast, co-expressing GFP-MGW with Flag-PAZ did not recruit the diffusely distributed Flag-PAZ to cytoplasmic GFP-MGW-positive foci. This data supported that the contention that interaction of GW182 with the C-terminal half of Ago2 mediated the localization of Ago2 in GWB.

**Ago2 Bound to Multiple Non-overlapping GW-rich Regions of GW182**

Since the GW182 fragments, GW1Δ1 and MGW, were both shown to bind Ago2 and these two fragments have overlapping 186aa, it is possible that the overlapping region of GW182 (aa566–751) is the primary site for the GW182-Ago2 interaction. To examine this possibility, deletion constructs GW1Δ1a (aa254–503) and GW1Δ1b (aa502–751) were generated with the
latter covering the overlapping region of GW1Δ1 and MGW (Fig. 4-1A). In addition, other deletion constructs GW1Δ7 (aa1034–1962) and GW1Δ5 (aa1670–1962) were used to investigate whether regions of GW182 other than GW1Δ1 and MGW bound Ago2. GFP-GW1Δ1a, -GW1Δ1b, -GW1Δ7, or -GW1Δ5 was co-expressed with GST-tagged Ago2 fragment PIWI in HeLa cells and a GST pull-down assay was performed to examine the interaction. As a negative control, GFP-GW1Δ1 was co-expressed with GST-tagged fragment N1, the N-terminal aa51–779 of a completely unrelated protein hZW10 (Famulski et al., 2008). Unexpectedly, GFP-GW1Δ1a, -GW1Δ1b, -GW1Δ7, and -GW1Δ5 all co-precipitated with GST-PIWI (Fig. 4-4). Interestingly, GW1Δ1a, GW1Δ1b, and GW1Δ5 are non-overlapping fragments and thus this data showed that at least 3 separate regions of GW182 could bind Ago2. Moreover, the Ago2-binding deletion constructs all contain GW-rich region whereas TNR, the only deletion construct that did not bind Ago2 lacked a GW-rich region. In summary, the GW182 deletion constructs containing GW-rich regions all bound to the C-terminal half of Ago2 indicating that multiple regions of GW182 mediated the interaction of GW182 with Ago2 and that GW repeat might be an key element for Ago2-binding.

The Interaction of Ago2 with GW182 Was Conserved in Other Human Ago Proteins

There are four Ago proteins in human that share a high degree of sequence similarity. To examine whether Ago1, Ago3, and Ago4 interact with GW182, GFP-Ago1, -Ago3, -Ago3m, or -Ago4 was co-expressed with GST-tagged GW182 fragments GW1Δ1 or MGW in HeLa cells and a GST pull-down assay was performed. Ago3m is a splicing variant of Ago3 and is missing aa757–823, the C-terminal 66aa of PIWI domain. Interestingly, human Ago1, Ago3, and Ago4 bound GW1Δ1 and MGW (Fig. 4-5). Ago3m did not bind GW1Δ1 or MGW indicating that the C-terminus of PIWI domain was required for the binding to GW182 (Fig. 4-5). Notably, both
GFP-Ago1 and Flag-Ago1 bound GST-GW1Δ1 demonstrating that different N-terminal fusion tags did not affect the binding of Ago1 with GW182. In summary, the interaction of human GW182 with Ago2 was observed with other human Ago proteins and the C-terminal region of PIWI domain was critical for the interaction of GW182 with Ago3.

**Tethering C-terminal Half of Ago2 to the 3’-UTR of mRNA Recapitulated Ago2-mediated Silencing Which Required GW182**

It was reported that tethering Ago2 to the 3’-UTR of mRNA causes repression of protein synthesis (Pillai *et al.* 2004). Since GW182 and Ago2 are stably associated with each other, the GW182-Ago2 interaction might help Ago2 mediate silencing through interaction with 3’-UTR of mRNA. Because the C-terminal half of Ago2 was shown to bind GW182 whereas the N-terminal half of Ago2 was not, we examined whether the C-terminal half of Ago2 was able to mediate silencing when tethered to the 3’-UTR of mRNA. The dual luciferase and tethering assay was used as described previously (Pillai *et al.*, 2004). In this assay, the reporter Renilla luciferase (RL) contains five 19-nt BoxB hairpin structures in the 3’-UTR of its mRNA (RL-5BoxB). The λN peptide, which is derived from λ phage and binds to BoxB structures with high affinity (Legault *et al.*, 1998), was fused to the N-terminus of Ago2, PIWI, and PAZ. In this way, Ago2, PIWI, or PAZ was brought directly to the 3’-UTR of mRNA bypassing the requirement for miRNA. Interestingly, tethered PIWI was attended by almost as much repression as tethered full-length Ago2 was (Fig. 4-6A). In contrast, tethered PAZ was totally devoid of the repression function of Ago2 (Fig. 4-6A). This data indicated that the functional domain mediating silencing lie within the C-terminal half of Ago2. To examine whether GW182 is required for Ago2- or PIWI-mediated repression, siRNA was used to knockdown GW182 before Ago2, PIWI or PAZ was tethered to the reporter mRNA. Very interestingly, both Ago2- and PIWI-mediated repression was significantly reduced upon GW182-knockdown (Fig. 4-6B).
The GW182-knockdown was confirmed by quantitative real time PCR (Fig. 4-6C). In summary, tethering the C-terminal half of Ago2 to the 3’-UTR of mRNA recapitulated the repression function of Ago2 and this repression required GW182.

**Discussion**

**Formation of GW182 and Ago Protein Complexes**

Two studies have identified that one GW-rich region capable of binding Ago2 is conserved in the plant and yeast orthologs of GW182, and that the GW repeat within this region is critical for GW182-Ago2 interaction (El-Shami et al., 2007; Till et al., 2007). Consistent with these studies, our data showed that human GW-rich regions were important for Ago2-binding (Fig. 4-7). GW182 fragments MGW (aa566–1343) and GW1Δ7 (aa1034–1962) containing the ortholog-conserved GW-rich region (aa1074–1144) (Till et al., 2007) were shown to bind Ago2 (Fig. 4-1A). In addition, our data showed that at least three non-overlapping regions of GW182 could independently bind Ago2 and, interestingly, these Ago2-binding fragments are outside of the ortholog-conserved GW-rich region (Fig. 4-7). Sequence alignment analysis showed that 27aa and 23aa residues of the ortholog-conserved GW-rich region shared 40.7% and 34.8% identity with the GW1Δ5 and GW1Δ1b, respectively (Fig. 4-7). However, significant sequence identity was not identified between the ortholog-conserved region and GW1Δ1a. The precise amino acid requirement for Ago2-binding remains unclear and requires further investigation. Nevertheless, our data lead to the speculation that one GW182 protein can bind multiple Ago proteins and this may contribute to the formation of functional translational silencing complexes. Since GW182 interacted with all four Ago proteins, it is possible that different Ago proteins incorporate into the same complex. The function of the silencing complex might depend on which Ago proteins it contains. It was reported more closely-spaced miRNA binding sites in the
3’-UTR of target mRNA lead to more efficient miRNA-mediated translational repression (Grimson et al., 2007). This supports our speculation that GW182 helps to stabilize the binding of multiple Ago-miRNA complexes to the 3’-UTR of target mRNA for more efficient translational repression. It is also possible that GW182 simultaneously binds to Ago-miRNA complexes on several different mRNAs and this GW182-Ago interaction may be the driving force for the assembly of submicroscopic and microscopic GWB. This hypothesis is supported by current observations that the GW1Δ1 GW182 fragment or the PIWI Ago2 fragment could mediate GW182-Ago2 interaction and by our previous data that overexpression of either of these two constructs disassembled GWB, possibly due to disruption of GW182-Ago2 interaction by a dominant-negative effect (Jakymiw et al., 2005).

**C-terminal Half of Ago2 Preserved the Silencing Function of Ago2 Probably Because It Maintained the Interaction with GW182**

Our GW182-Ago2 interaction mapping showed that the C-terminal half of Ago2 (aa478–860) was sufficient for the binding with GW182 whereas N-terminal half (aa1–480) was not required. Interestingly, only the C-terminal half of Ago2 preserved the silencing function of Ago2 when directly brought to the 3’-UTR of target mRNA. The silencing function mediated by Ago2 or the C-terminal half of Ago2 was abolished upon GW182-knockdown. Our data strongly suggested that interaction of Ago2 with GW182 is critical for the silencing process mediated by Ago2 at the 3’-UTR of target mRNA. This hypothesis is also supported by two recent studies where overexpressing the Ago-binding fragment of yeast or Drosophila ortholog of GW182 greatly disrupted GW182-Ago interaction and significantly impaired miRNA-mediated silencing in vitro and in vivo (Till et al., 2007; Eulalio et al., 2008).

Our data also suggested that Ago2 is not the “final repressor” because its silencing function relied greatly on GW182. GW182 probably functions downstream of Ago proteins and mediates
translational repression one step further. Interestingly, the PIWI domain of Ago2 was reported to be responsible for the interaction with Dicer (Tahbaz et al., 2004). It is intriguing to postulate that Dicer and GW182 may compete for the binding with Ago2 through the PIWI domain. Notably, tethering the N-terminal half of Ago2 to the 3’-UTR of mRNA seemed to upregulate protein levels. A previous study showed that tethering the PAZ domain alone or the smaller N-terminal fragment of Ago2 to the 3’-UTR of mRNA did not upregulate translation (Pillai et al., 2004). This discrepancy may be explained by the difference in the deletion constructs used in these studies. Our data lead to the speculation that there may be an “activation domain” in the N-terminal half of Ago2 that could upregulate protein synthesis and may explain how Ago2-miRNA complex can activate translation under certain circumstances (Bhattacharyya et al., 2006; Vasudevan et al., 2007; Buchan and Parker, 2007). However, defining this activation domain and how it activates translation needs further investigation.

Based on the data from current study, we propose a model for miRNA-mediated gene silencing in which, after miRNA guides Ago-miRNA complex to the 3’-UTR of target mRNA, Ago protein recruits GW182 to stabilize Ago-miRNA-mRNA binding and represses translation. In addition, GW182-Ago2 interaction recruits Ago2 to GWB, which accumulate many Ago2-miRNA-mRNA complexes and become centers for miRNA-mediated silencing.

*This work was submitted to Journal RNA for publication and is in revision now.
Figure 4-1. Identifying the interaction of C-terminal half of Ago2 with GW182 fragments using GST pull-down assays. A) Schematic of human GW182 and Ago2 deletion constructs. All amino acid residues are referenced to the longer isoform of GW182 (GenBank Accession NM_014494.2). Q-repeat, glutamine repeat (box in white); Q/N-rich, glutamine/asparagine-rich region (box in magenta); RRM, RNA recognition motif (box in green); GW-rich, glycine/tryptophan-rich region (boxes in yellow); N-GW, N-terminal GW-rich region; M-GW, middle GW-rich region; C-GW, C-terminal GW-rich region. Human Ago2 is mainly comprised of two domains: PAZ domain (box in blue) and PIWI domain (box in red). B) Endogenous Ago2 co-precipitated with GW182 fragments GW1Δ1 (aa254–751) and MGW (aa566–1343) but not with TNR (aa1–204). GST-tagged TNR (lanes 1,4), GW1Δ1 (lanes 2,5), or MGW (lanes 3,6) was transfected into HeLa cells. Endogenous Ago2 was detected by using rabbit anti-Ago2. In a longer exposure (lanes 7–9), Ago2 was detected more clearly being co-precipitated with GST-MGW (lane 9) but still absent in GST-TNR precipitates (lane 7). C) GW182 fragments GW1Δ1 and MGW co-precipitated with Ago2 fragment PIWI (aa478–860) but not with PAZ (aa1–480). GST-MGW (lanes 1–6) or GST-GW1Δ1 (lanes 7–10) was co-transfected with Flag-tagged Ago2, PIWI, or PAZ. Full-length Ago2 (lanes 4,9) and PIWI (lanes 5,10), but not PAZ (lane 6),
co-precipitated with GST-MGW or GST-GW1Δ1. D) GW182 fragment TNR did not pull-down Ago2. Flag-Ago2 was co-transfected with GST-tagged TNR (aa1 – 204, lanes 1, 3) or with GW1Δ1 (lanes 2, 4) as a positive control.
Figure 4-2. The GW182 fragment MGW formed insoluble complexes with Ago2 and C-terminal half of Ago2. GST-MGW was co-transfected with Flag-Ago2, -PIWI, or -PAZ in HeLa cells. The expression of GST-MGW in total cell lysate was relatively uniform (lanes 1–3). Higher level of GST-MGW was detected in the insoluble fraction when co-transfected with Flag-Ago2 (lane 4) or Flag-PIWI (lane 5) whereas GST-MGW was barely detectable when co-transfected with Flag-PAZ (lane 6). The level of tubulin served as a loading control.
Figure 4-3. GW182 fragment MGW recruited Ago2 to cytoplasmic foci by interacting with the C-terminal half of Ago2. GFP-MGW (green, a-c) was co-transfected with Flag-Ago2 (d), PIWI (aa478 – 860, f) or PAZ (aa1 – 480, h) into HeLa cells. As controls, Flag-PIWI (e) or Flag-PAZ (g) was singly transfected. The cells were stained with anti-Flag antibody (red, d-h). Panels in the bottom row are the merged images (i-m). Nuclei were counterstained with DAPI (blue). Scale bar, 10μM.
Figure 4-4. The C-terminal half of Ago2 bound to multiple non-overlapping GW-rich regions of GW182. GST-PIWI (aa478–860) was co-transfected with GFP-GW1Δ1 (lane 1), -GW1Δ1a (lane 2), -GW1Δ1b (lane 3), -GW1Δ7 (lane 4), -TNR (lane 11), or -GW1Δ5 (lane 12) into HeLa cells. Similar to positive control GW1Δ1 (lane 6), GW1Δ1a (lane 7), GW1Δ1b (lane 8), GW1Δ7 (lane 9), GFP-GW1Δ5 (lane 14), but no GFP-TNR (lane 13), were detected in GST-PIWI precipitates. GST-tagged N1, N-terminal fragment from an unrelated protein hZW10, was co-transfected with GFP-GW1Δ1 (lane 5) as a negative control and no interaction was detected (lane 10).
Figure 4-5. Both GW182 fragments GW1Δ1 and MGW co-precipitated with other human Ago proteins.  A) Ago 1 and Ago4, but not Ago3 mutant, co-precipitated with GW182 fragments GW1Δ1 and MGW.  GFP-Ago1, -Ago3m (Ago3 mutant), or -Ago4 was co-transfected with GST-MGW (lanes 1–6) or GST-GW1Δ1 (lanes 7–12).  Ago3m is missing aa757 – 823, the C-terminal 66aa of the PIWI domain.  Both Ago1 (lanes 4,10) and Ago4 (lanes 6,12) were pulled down by GST-MGW or GST-GW1Δ1.  In comparison, Ago3m was absent from either pull-down (lanes 5,11).  B) Ago3 co-precipitated with GW182 fragment GW1Δ1.  Flag-Ago3 (lanes 1,3) or -Ago1 (lanes 2,4) was co-transfected with GST-GW1Δ1 (lanes 1–4).  GFP-Ago3m (lanes 5–6) was co-transfected with GST-GW1Δ1 as a negative control.
Figure 4-6. Translational repression mediated by tethered C-terminal half of Ago2 required GW182.  A) Tethered PIWI (aa478–860) down-regulated protein synthesis.  HeLa cells were transfected with constructs expressing the RL-5BoxB reporter, control FL reporter, and indicated NHA-tagged proteins.  Bar graphs represent normalized mean values of RL/FL activities with standard errors.  The RL/FL values in cells with tethered NHA were normalized as 1.  The expression of fusion proteins was determined by Western Blot using anti-HA mAb and are indicated below the bar graphs.  The assay was performed in triplicates and was repeated for at least 3 times.  *significant difference (unpaired t test, p<0.01); ns, no significant difference (unpaired t test, p>0.05).  B) Translational repression mediated by tethered Ago2 or PIWI (aa478–860) was greatly impaired upon GW182-knockdown.  HeLa cells were transfected with siRNA for either GW182 (siGW182) or GFP (siGFP).  Thirty hours later, cells were transfected again with constructs expressing reporter RL-5BoxB, control FL reporter, and the same NHA-tagged proteins as indicated in panel A.  Bar graphs represent the reduction of RL/FL in cells with tethered NHA-Ago2 or NHA-PIWI compared to those in cells with tethered NHA.  The reduced values of RL/FL in cells transfected with GFP-siRNA were set as 1.  Error bars indicate standard errors.  The assay was performed in triplicates and was repeated for 2 times.  *significant difference (unpaired t test, p<0.01).  C) GW182-knockdown by siRNA was confirmed by quantitative real time PCR.  The bar graphs represent normalized mRNA level of GW182 with standard errors.  The mRNA level of GW182 in cells transfected with GFP-siRNA was set as 1.  The experiment was performed in triplicates.
Figure 4-7. At least three non-overlapping GW-rich regions that are different from the ortholog-conserved GW-rich region can independently bind Ago2. The dot graph on top indicates the distribution of tryptophan in GW182. W, every tryptophan (magenta diamond); WG/GW, a glycine right adjacent to tryptophan (red triangle); AW/WA, an alanine right adjacent to tryptophan (blue diamond); W only, no glycine or alanine right adjacent to tryptophan (green diamond). The majority of the tryptophans are adjacent to either a glycine or alanine. The schematic of GW182 is indicated below the dot graph. GW-rich region, box in yellow; ortholog-conserved GW-rich region (aa1074–1144, (Till et al., 2007)) box in red. GW1Δ1a, GW1Δ1b and GW1Δ5 are the three non-overlapping regions identified in the current study that binds Ago2. The amino acid sequence alignment between GW1Δ5/GW1Δ1b and ortholog-conserved GW-rich region was performed using ExPASy website tool. * indicating identical conservation.
GW/P Body Is a Processing Center for Messenger RNAs Targeted by RNAi Pathway with Its Component GW182 Playing a Critical Role in the Silencing Process

Two interesting studies (Chu and Rana, 2006; Eulalio et al., 2007b) have been published after our report showing that the integrity of microscopic-detectable GWB is dispensable for RNAi (Jakymiw et al., 2005). In these two studies, disruption of GWB by knocking down decapping factor LSm proteins, which are important for GWB formation, does not affect siRNA- or miRNA-mediated silencing. Nevertheless, one of the studies showed that blockage at any step of siRNA or miRNA pathway leads to disappearance of GWB and that transfecting siRNA into Drosha-knockdown cell reforms the foci (Eulalio et al., 2007b). These data are consistent with another study from our laboratory (Pauley et al., 2006) and support that formation of GWB is the consequence of siRNA- and miRNA-mediated silencing. Furthermore, the current studies show that the number and size of GWB increase when siRNA silencing is activated. Knockdown of Ago2 impairs siRNA silencing and prevents the increase of GWB. Interestingly, although knockdown of rck/p54 or LSm1 disassembles GWB, they do not prevent the reformation of GWB since the siRNA silencing activity is intact (Lian et al., 2007). Altogether, these recent studies indicate that, although the microscopic-detectable GWB are not required for the initiation of siRNA/miRNA silencing, siRNA:mRNA or miRNA:mRNA duplex activates the formation of GWB as long as the RNAi pathway is intact, supporting that GWB are structures for storing and/or processing the sequestered mRNAs targeted by siRNA or miRNA. This is consistent with the observed high enrichment of siRNAs, miRNAs, and target mRNAs in GWB. It is unclear whether the siRNA:mRNA and miRNA:mRNA duplexes require submicroscopic GWB for docking, or they could recruit decay factors to form de novo GWB. Why GWB form and whether the formation of GWB makes the subsequent mRNA degradation step more efficient are
unknown. To address these possibilities and questions needs further investigations. It is noted that a recent study reported an individual siRNA for CD81, a transmembrane protein apparently unimportant for GWB formation, surprisingly causes disassembly of GWB (Serman et al., 2007). However, other two different siRNAs for the same protein do not. This suggests that this particular siRNA may have off-target effects and may have targeted important genes in RNAi pathway.

Since the importance of GWB for RNAi is controversial, we reexamined how disruption of GWB impairs RNAi function in our initial study (Jakymiw et al., 2005) based on the data from the current studies. The three methods we use to disrupt GWB, including expression of dominant negative constructs GW1Δ1 and Ago2 PIWI (aa478–860), and knockdown of GW182, all apparently interfere with the GW182-Ago2 interaction that has been proven important for miRNA function in human by the current studies and in Drosophila by two other studies (Till et al., 2007; Eulalio et al., 2008). The interaction of GW182 with Ago2 possibly recruits GW182 to target mRNA to critically repress translation. In addition, the GW182-Ago2 interaction may recruit RISC/miRNP-bound target mRNAs to GWB for processing or cause them to aggregate into GWB. The disassembly of GWB is probably the consequence of disruption in GW182-Ago2 interaction and impairment in miRNA function. The implication from our initial study is that, potentially, the GW182-Ago2 interaction is required for siRNA-mediated silencing. However, the exact function of this interaction in siRNA function needs to be further confirmed and investigated.

**Working Model and Conclusions**

We propose a working model that illustrates the important advance in understanding how GW182 and GW/P body involved in RNAi attributed by our current work (Fig. 5-1). Our
findings define for the first time GW182 as a potential translation repressor working downstream of Ago proteins. The GW182-Ago interaction may contribute to the aggregation of translation repressed mRNAs and the formation of GWB. Although the formation of GWB may not be required for initial RNAi activity, active RNA silencing initiates the assembly of GWB which therefore become processing centers for mRNA targets and biomarkers for RNAi activity. The work presented here advance the molecular and cell biology of RNAi, and may provide insight into the future application and monitoring of RNAi.

**GW/P Body May Regulate Multiple Cellular Processes**

Factors involved in several different cellular pathways have been identified as components of GWB (Tab. 5-1). In addition to mRNA decay/storage/transportation and RNAi, recently GWB have been suggested to associate with viral life cycle and innate anti-viral defense (Beckham and Parker, 2008). The initial evidence from the analysis of yeast retrotransposons Ty1 and Ty3 shows that yeast P body component LSm1-7p complex and Dhh1p (ortholog of rck/p54) are required for efficient retrotransposition for both Ty1 and Ty3 (Griffith et al., 2003).

In addition, deletion in the deadenylase complex Ccr4/Pop2 results in enhanced retrotransposition (Irwin et al., 2005). Since in yeast LSm1-7p and Dhh1p promote the formation of P body, and Ccr4p/Pop2p limits P body formation (Coller and Parker, 2005; Teixeira and Parker, 2007), these observations suggest that targeting of Ty transcripts to P bodies might be important for retrotransposition. Another evidence is that the human RNA helicase DDX3 is required for the export and translation of unspliced HIV-1 RNA from the nucleus (Yedavalli et al., 2004). Interestingly, the yeast ortholog of DDX3, Ded1p, is shown to accumulate in P bodies and is important for their formation (Beckham et al., 2008). This suggests that in human cells DDX3 may recruit the unspliced HIV-1 RNA, which serves as genomic RNA of HIV-1, to GW/P body for subsequent steps in viral function. Furthermore,
GWB are also linked to anti-viral defense by the evidence that the anti-viral proteins APOBEC3G and APOBEC3F are concentrated in GWB, and that APOBEC3G interacts with Ago1 and Ago2 (Wichroski et al., 2006; Gallois-Montbrun et al., 2007). Members of APOBEC (apolipoprotein B RNA-editing enzyme catalytic polypeptide 1-like) family of cytidine deaminases are thought to play an anti-viral role by preventing host cell genome from invasion by retroviruses or retrotransposons (Wedekind et al., 2003). Interestingly, the Vif protein of HIV-1, which binds to APOBEC3G and triggers its degradation, is found to localize to GWB in an APOBEC3G-dependent manner (Wichroski et al., 2006). These observations suggest that APOBEC3G and APOBEC3F might function in the microenvironment of GWB to restrict HIV-1 replication.

As important components of GWB, miRNAs has been shown to play a critical role in cell cycle, cell proliferation and tumor genesis (Lian et al., 2006; Kent and Mendell, 2006; Carleton et al., 2007). More than 10 miRNAs have been identified to target factors, such as Bcl-2, Ras, and E2F1, which are critical for cell cycle progression and cellular proliferation (O'Donnell et al., 2005; Kent and Mendell, 2006). Moreover, proto-oncogene c-Myc or tumor suppressor p53 is shown to regulate the level of specific miRNAs (He et al., 2005; O'Donnell et al., 2005; He et al., 2007a; He et al., 2007b). Alteration of miRNA levels can contribute to pathological conditions, including tumorigenesis, which are associated with loss of cell cycle control (Kent and Mendell, 2006). Interestingly, GWB have been shown to vary in number and size at different stage of cell cycle and during cell proliferation (Yang et al., 2004). All these evidence support that GWB are potentially important sites for miRNA-mediated regulation of these cellular mechanisms.

Two recent studies from our laboratory suggest that GWB may be associated with autoimmune disease. The first study reports that a set of autoantibodies from patients with
rheumatic diseases and in a mouse model of autoimmunity recognize key components of RNAi including Ago1-4 and Dicer (Jakymiw et al., 2006). It implicates the potential involvement of RNAi pathway in the pathogenesis of autoimmune diseases. This is also supported by another recent study which shows that misregulation of a single miRNA leads to systemic lupus (Yu et al., 2007). The second study from our laboratory shows that miRNA regulates human monocyte functions such as cytokine and chemokine production via the formation of GW/P body (Pauley et al., 2008). The overproduction of inflammatory cytokines and chemokines have been shown to closely related with autoimmune diseases such as systemic lupus erythematosus, Sjögren’s syndrome and rheumatoid arthritis.

The latest advance of RNAi pathway is the discovery of endogenous siRNAs (esiRNAs) in Drosophila and mouse. Investigators used to think that esiRNAs only existed in organisms that possess RNA-dependent RNA polymerases (RDRPs), such as plants, C. elegans, and yeast. However, seven studies published recently totally change this concept (Czech et al., 2008; Kawamura et al., 2008; Tam et al., 2008; Watanabe et al., 2008; Ghildiyal et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). These studies uncover that esiRNAs surprisingly exist in both Drosophila and mouse oocytes, and that the esiRNAs play a role in suppressing the expression of retrotransposons and in regulating specific protein-encoding transcripts complementary to them (Tam et al., 2008; Watanabe et al., 2008). Most of the identified esiRNAs are derived from a variety of sources: long hairpin structures, convergent transcription, bidirectional transcription, and transposable elements (Nilsen, 2008). Interestingly, some of the esiRNAs in mouse oocytes are processed from overlapping regions of encoding genes and cognate pseudogenes (Tam et al., 2008; Watanabe et al., 2008). These findings suggest that pseudogenes may actually regulate the expression of their founder gene. Although
the extent and biological relevance of esiRNAs await further investigation, discovery of esiRNAs as a new class of small RNAs broadens the scope of regulatory networks mediated by small RNAs. Since both Drosophila and mouse that do not have RDRPs can generate esiRNAs, it is not surprising that esiRNAs will be identified in human cells. It would be interesting to further investigate the possible existence of and the potential functions of esiRNAs in human cells. Since esiRNAs are structurally the same as exogenous siRNA, we speculate that esiRNAs may be also enriched in GWB as siRNA, and that their functions closely associate with GWB as well.

Taken together, GWB are directly or indirectly involved in multiple cellular pathways (Fig. 5-2). It is intriguing to postulate that the driving force for these pathways correlating with GWB is that certain steps of them may be regulated by siRNA or miRNA. The formation of GWB physically sequesters the regulated mRNAs from polysomes which may help to quickly and efficiently stop translation. The translation repressed mRNAs are stored and degraded in the GWB, and may go back to translation under certain circumstance. However, the exact function of GWB in these pathways needs further investigations.
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Figure 5-1. A proposed model of the function of GW182 and GW/P body in RNAi. In siRNA pathway (upper left), siRNA bound to Ago2 forming active RISC which then binds to and cleaves target mRNA with complete complementary sequence. The interaction of GW182 with Ago2 recruits the cleaved mRNA to GW/P body for degradation. In miRNA pathway (upper right), miRNA binds Ago protein and forms miRNP, which subsequently binds to the 3’-UTR of mRNA with incomplete complementary sequence (boxes in orange on mRNA). However, the binding of miRNP to mRNA is not able to stop translation until GW182 is recruited. GW182 possibly help to stabilize the binding of miRNPs to the 3’-UTR and is the repressor for translation. The translation repressed mRNA is recruited to GW/P body for storage and/or degradation. Under certain circumstance, the stored repressed mRNA can go back to translation. The GW182-Ago protein interaction helps the cleaved and translation repressed mRNAs aggregate into GW/P body where mRNA decay factors (Pacman) are recruited to degrade the messages. Under the circumstance that siRNA- and/or miRNA-mediated silencing are activated, more target mRNAs are recruited to GWB for processing leading to an increase in the number and the size of GWB.
Figure 5-2. Multiple cellular pathways associated with GWB. The image in the middle is an immunogold electron microscope image of GW body. Arrows point to the cellular pathways that are considered to be associated with GWB. Dotted arrows point to the two cellular processes that have been shown regulated by miRNA silencing. The key references proving the links between these pathways and GWB are listed as following: miRNA silencing (Liu et al., 2005b), siRNA silencing (Jakymiw et al., 2005), mRNA decay (Sheth and Parker, 2003), mRNA storage (Brengues et al., 2005), viral life cycle (Beckham and Parker, 2008), anti-viral defense (Wichroski et al., 2006; Gallois-Montbrun et al., 2007), autoimmune disease (Jakymiw et al., 2006), cell proliferation (Yang et al., 2004), cell cycle (Lian et al., 2006; Kent and Mendell, 2006), and innate immune signaling (Pauley et al., 2008).


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BIOGRAPHICAL SKETCH

Shang Li Lian was born on Dec 7, 1977, in Guangzhou, China. She studied clinical medicine at Sun Yat-sen University, a famous medical school in China, and received Bachelor of Medicine in 2002. Inspired by her father, Shang got interested in biomedical research which helped her further understand medical science from a brand new angle. She was admitted and entered the Interdisciplinary Program in Biomedical Sciences in College of Medicine at the University of Florida in 2003. Shang joined the laboratory of Dr. Edward K.L. Chan in the summer of 2004. She began her research on the function of GW182 and GW/P body in RNA interference. She received Ph.D. degree in Medical Sciences-Molecular Cell Biology in Aug 2008. Shang planned to continue her research on the cell biology of RNA interference. Her ultimate goal is to become a pathologist and eventually start her own laboratory. During her Ph.D. study, Shang was married to Songqing Li in Oct 2004.