Formation of GW bodies is a consequence of microRNA genesis

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GW bodies (GWBs), or mammalian P bodies, proposed to be involved in messenger RNA storage and/or degradation, have recently been linked to RNA interference and microRNA (miRNA) processing. We report that endogenous *let-7* miRNA co-precipitates with the GW182 protein complex. In addition, knockdown of two proteins, Drosha and its protein partner DGCR8, which are vital to the generation of mature miRNA, results in the loss of GWBs. Subsequent introduction of short interference RNA specific to lamin A/C is accompanied by reassembly of GWBs and concurrent knockdown of lamin A/C protein. Taken together, these studies show that miRNAs are crucial components in GWB formation.

Keywords: human autoantigen GW182; short interference RNA; microRNA; GW bodies; RNA interference

EMBO reports (2006) 7, 904–910. doi:10.1038/sj.embor.7400783

INTRODUCTION

In the past few years, unique cytoplasmic foci have been described in both yeast and mammalian cells that have a role in messenger RNA processing and degradation. They have been named P bodies in yeast (Sheth & Parker, 2003; Kshirsagar & Parker, 2004; Teixeira *et al*, 2005), and in mammalian cells they have been named Dcp-containing bodies (Van Dijk *et al*, 2002; Cougot *et al*, 2004), GW bodies (GWBs; Eystathiou *et al*, 2002) or mammalian P bodies (Liu *et al*, 2005b). In this report, we refer to the mammalian cytoplasmic foci as GWBs. GWBs were initially named as such because they represented the cytoplasmic localization of the mRNA-binding protein GW182. GW182 has repetitive glycine/tryptophan residues and a canonical RNA recognition motif, and was shown to associate with certain

mRNAs in HeLa cells (Eystathiou *et al*, 2002). The GW182 protein is a salient feature of GWBs in that reduction of the GW182 protein leads to a decline in the number of GWBs (Yang *et al*, 2004). GW182 is conserved among mammalian cells and, recently, a protein called Argonaute interacting protein 1 (AIN-1), with limited sequence similarity to GW182, was identified in *Caenorhabditis elegans* and shown to interact with components of the RNA-induced silencing complex (RISC; Ding *et al*, 2005). Yeast P bodies and mammalian GWBs are similar in that they are both composed of proteins involved in mRNA degradation, nonsense-mediated decay and translation (Bashkirov *et al*, 1997; Ingelfinger *et al*, 2002; Eystathiou *et al*, 2003; Sheth & Parker, 2003; Cougot *et al*, 2004; Unterholzner & Izaurralde, 2004; Andrei *et al*, 2005; Fukuhara *et al*, 2005; Kedersha *et al*, 2005). However, key differences include the observations that there is no protein with significant sequence homology or identity to GW182 in yeast, and GWBs have been linked to RNA interference (RNAi; Jakymiw *et al*, 2005), a pathway that has yet to be described in *Saccharomyces cerevisiae*.

GWBs seem to be involved in RNAi, from the observation that Argonaute 2 (Ago2), short interference RNA (siRNA), microRNA (miRNA) and reporter mRNAs targeted by miRNA localize to these bodies (Jakymiw *et al*, 2005; Liu *et al*, 2005b; Pillai *et al*, 2005; Sen & Blau, 2005). Furthermore, the GW182 protein has been shown to have an important functional role in RNAi (Jakymiw *et al*, 2005; Liu *et al*, 2005a; Rehwinkel *et al*, 2005; Behm-Ansmant *et al*, 2006). Although GWBs are implicated in the miRNA pathway, the functional requirement of miRNA in GWB formation remains unclear.

MiRNAs are small, 21- to 23-nucleotide non-coding RNAs that target specific mRNAs for either degradation or the prevention of its translation into protein by the RNAi pathway (Tang, 2005). Genes encoding miRNA are transcribed by RNA polymerase II (Pol II) to generate the primary miRNA transcript (pri-miRNA), which is capped and polyadenylated (Cai *et al*, 2004; Lee *et al*, 2004). In the nucleus, an RNase III enzyme known as Drosha, in conjunction with its partner DGCR8 in mammals, or its equivalent Pasha in *Drosophila*, cleaves the pri-miRNA transcript producing an approximately 70-mer hairpin pre-miRNA structure, which is then transported to the cytoplasm through exportin 5 (Lee *et al*,

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Fig 1 | MicroRNA is present in GW bodies. (A) Transfected *let-7* microRNA (miRNA) 3'-labelled with Cy3 was localized to GW bodies (GWBs) in HeLa cells 24 h after transfection. HeLa cells were transiently transfected with Cy3-3'-*let-7* after which they were fixed and stained with human anti-GWB serum. Analysis showed that the miRNA (i, arrows) was found within GWBs (ii, arrows). Nuclei were counterstained by 4,6-diamidino-2-phenylindole (DAPI; iii). Note that miRNA-transfected cells often show large cytoplasmic aggregates (i, arrowheads) distinct from GWBs (ii, arrows). The merged images are seen in (iv). Scale bar (i), 10 μ m. (B) Biochemical detection of *let-7* RNA using an RNase protection assay (upper) and GW182 protein by western blot analysis (lower, lane 2). Lanes 1 and 3 are control immunoprecipitates (IP) with normal human serum (NHS), and total RNA and protein from HeLa cell extracts, respectively.

2003; Yi *et al*, 2003; Denli *et al*, 2004; Gregory *et al*, 2004; Han *et al*, 2004; Lund *et al*, 2004; Tomari & Zamore, 2005).

In human cells, studies have shown that knockdown of Drosha results in a sharp decline of pre-miRNA and miRNA accompanied by the accumulation of pri-miRNA (Lee *et al*, 2003; Gregory *et al*, 2004; Han *et al*, 2004; Landthaler *et al*, 2004). Furthermore, knockdown of DGCR8 with siRNA also results in a sharp reduction in the amount of pre-miRNAs and hence mature miRNAs (Gregory *et al*, 2004; Han *et al*, 2004; Landthaler *et al*, 2004). Taken together, these studies show the importance of Drosha and DGCR8 in the generation of mature miRNA.

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

RESULTS AND DISCUSSION

We transfected HeLa cells with Cy3-3'-labelled *let-7* miRNA, to observe visually the intracellular localization of miRNA. We observed that the Cy3-labelled miRNA was located mainly in discrete foci that colocalized with GWBs by indirect immunofluorescence (IIF; Fig 1A). These transfection data are consistent

with a recent report showing that HeLa nuclei microinjected with *in vitro*-transcribed Cy3-pre-*let-7* miRNA are found in or adjacent to mammalian P bodies (Pillai *et al*, 2005). To determine whether *let-7* was found naturally in GWBs, HeLa cell extracts were immunoprecipitated using GW182 antibodies. An RNase protection assay using a 32 P-labelled *let-7* probe was then performed to determine specifically whether endogenous *let-7* was localized to the immunoprecipitate. As expected, the immunoprecipitates contained *let-7*, whereas the product immunoprecipitated by normal human serum did not (Fig 1B). This result supported the observations made with the IIF data described above.

Next, we questioned the importance of miRNA in GWB formation and whether the disruption of mature miRNA production would generate a phenotype associated with GWBs. Our approach was based on the published strategy that knockdown of Drosha or DGCR8 resulted in a reduction in the amount of pre-miRNA and mature miRNA after 72 h (Gregory *et al*, 2004; Han *et al*, 2004; Landthaler *et al*, 2004). As a result, HeLa cells were transiently transfected with a plasmid encoding a short hairpin RNA (shRNA) targeting Drosha (pDrosha-sh), to reduce Drosha protein through RNAi. Ten other unrelated plasmids, including one encoding shRNA to nuclear pre-lamin A recognition factor (pNarf-sh), were also transfected into HeLa cells as controls for

Fig 2 | Loss of GW body staining in Drosha-deficient HeLa cells and transfection of synthetic short interference RNAs rescues GW bodies. HeLa cells transfected with pDrosha-sh plasmid were allowed to grow in the presence of 2 μ g/ml puromycin to select for cells that were later transfected with lamin A/C short interference RNA (siRNA). (A) Untreated HeLa cells, (B) Drosha-deficient cells selected for 14–21 days, and (C) these cells 48 h after transfection with siRNA were co-stained with GW182 antibody and Alexa 488-goat anti-human IgG (green, i,iv,vii), and lamin A/C monoclonal antibody and Alexa 350-goat anti-mouse IgG (blue, ii,v,viii). GW bodies (GWBs; arrows) were readily detected in untreated HeLa cells (i). Most of the GWB staining was lost in the Drosha-deficient cells, but dispersed residual GWB staining was still apparent (arrows, iv). Many GWBs were observed in Drosha-deficient cells after transfection with siRNA (arrows, vii). The knockdown of lamin A/C in Drosha-deficient cells transfected with lamin A/C siRNA was effective (viii), with only few cells having detectable lamin A/C (arrowhead). (D) Cy3-labelled siRNA for lamin A/C localized to GWBs in transfected Drosha-deficient cells. Most of the Cy3-siRNA seemed to be aggregated in the cytoplasm but some was detected in GWBs (arrows). Efficient lamin A/C knockdown was observed and was indicated by the absence of lamin A/C staining in the nucleus (dashed circle). Insets are enlarged 1.5-fold and the Cy3 signal is enhanced to show the localization of siRNA to GWBs. Scale bars, 10 μ m.

any nonspecific effect associated with these plasmids. It is important to note that these shRNAs are transcribed in the nucleus and processed by the Drosha–DGCR8 complex in a manner similar to endogenous pri-miRNA. Thus, when Drosha or DGCR8 is knocked down by the shRNA-derived siRNA, processing and maturation of newly transcribed shRNAs, as well as endogenous pri-miRNAs, is inhibited resulting in a reduction in these RNA levels. As the transfection efficiency using the typical transfecting agent Lipofectamine2000 was around 20–25%, we co-transfected

a green fluorescent protein (GFP) expression plasmid (pGFP) such that transfected cells were identifiable. These cells were then stained for GWBs by IIF using human GWB antibodies (Eystathiou *et al*, 2002, 2003; Yang *et al*, 2004) and for Drosha protein by rabbit Drosha antibodies. Seventy-two hours after transfection, most of the cells co-transfected with pDrosha-sh and pGFP had a clear reduction in the number and size of GWBs (supplementary Fig 1iii online), and there was a strong correlation with the reduction in Drosha staining (supplementary Fig 1ii online).

Fig 3 | Characterization of Drosha-deficient cells and demonstration of reduced microRNA levels. (A) Reduction of messenger RNA levels of Drosha demonstrated by using reverse transcription-PCR (RT-PCR) to compare untreated HeLa cells and pDrosha-sh-transfected HeLa cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) analysis was included for comparison. (B) Reduction of mature microRNA (miRNA) levels demonstrated by using RT-PCR to compare untreated HeLa cells and pDrosha-sh-transfected cells. 5S RNA analysis was included for comparison. (C) Western blot analysis showing a reduction of Drosha protein level in pDrosha-sh-transfected cells (data also shown from duplicate experiment) compared with untreated HeLa cells. GW182, Argonaute 2 (Ago2), Rck/p54 and actin levels are shown for comparison.

Control cells transfected with phrGFP and pNarf-sh showed a normal distribution of GWBs and Drosha protein (supplementary Fig 1v–viii online). The Drosha-deficient cells were also stained with another P body marker, Dcp1a, which showed a phenotype that was identical to GWB staining (supplementary Fig 2 online).

However, as the transfection efficiency of the plasmid was only around 20% and the number and size of GWBs are known to vary during the cell cycle (Yang et al, 2004), we verified this observation by selecting pDrosha-sh-transfected cells by puromycin resistance. After 21 days of selection, we reproduced the phenotype obtained with the transiently transfected HeLa cells in that the number of GWBs sharply declined in the selected cells (Fig 2B, compare with Fig 2A). In a typical experiment, around 95% of selected cells (~1,000 cells) completely lacked GWBs, and the remaining 5% had one or two small GWBs. It was confirmed by RNA and protein analysis that Drosha protein was substantially reduced in these cells (Fig 3A,C) and, as predicted, mature miRNA levels were greatly reduced in these cells compared with untreated HeLa cells (Fig 3B). These results indicated that miRNA was central to GWB formation.

Next, we set out to examine whether the introduction of siRNA, a surrogate for miRNA, could rescue GWB formation. Transfection of the Drosha knockdown cells with lamin A/C siRNA resulted in the reappearance of GWBs in these cells in 48 h (compare Fig 2vii with 2iv), the localization of siRNA to GWBs (Fig 2D) and a markedly reduced expression of lamin A/C mRNA (supplementary Fig 3A online) and protein (Fig 2Cviii,D; supplementary Fig 3b online). HeLa cells and the Drosha knockdown cells had normal levels of lamin A/C mRNA and protein

Fig 4 | Disassembly of GW bodies in HeLa cells resulting from DGCR8 knockdown and reassembly after short interference RNA transfection. A short hairpin RNA plasmid targeting DGCR8 (pDGCR8-sh) was transiently transfected with the green fluorescent protein (GFP) expression plasmid (phrGFP) at a 5:1 molar ratio. After 72 h, transfected cells identified by the coexpression of GFP showed almost complete absence of GW body (GWB) staining, although a few weakly stained GWBs were detectable (arrows, vi). By contrast, GWBs were detected in cells transfected with phrGFP alone (arrows, iii). Transfection of short interference RNA (siRNA) for lamin A/C in DGCR8 knockdown cells resulted in a reappearance of GWBs (arrows, ix) and a reduction in lamin A/C protein in these cells 48 h after siRNA transfection. Scale bar, 10 μ m.

Fig 5 | GW bodies are foci specific to microRNA processing. In this schematic diagram, primary microRNA (pri-miRNA) nuclear transcripts are cleaved by the Drosha-DGCR8 complex into hairpin pre-miRNA, which is exported to the cytoplasm by exportin 5. In the cytoplasm, Dicer is responsible for cleavage of pre-miRNA, generating the mature miRNA. On correct interaction and partnering of the miRNA to messenger RNA containing the miRNA-targeted sequence element 'X' in the presence of Ago2/RISC (RNA-induced silencing complex), cytoplasmic foci are formed as a result of association of other proteins (see text). However, in the absence or low levels of miRNA, as a result of Drosha or DGCR8 protein knockdown, these foci do not form. The transfection of short interference RNA (siRNA) as a surrogate for miRNA leads to the apparent reassembly of GW bodies (GWBs). Ago2, Argonaute 2.

expression before the addition of lamin A/C siRNA (supplementary Fig 3A,B online). Transfection of chemically modified siRNA that impairs uptake and processing by RISC ('RISC-free' siRNA) into Drosha-deficient cells failed to rescue GWB formation (supplementary Fig 4 online).

To substantiate independently the observed reduction in the number of GWBs seen in Drosha-deficient cells, we transfected HeLa cells with two separate plasmids (pDGCR8-sh and pDGCR8-sh2) encoding shRNA directed to different sequences in the mRNA for the partner of the Drosha protein, DGCR8. Fig 4 shows cells that were transiently transfected with phrGFP alone or with both phrGFP and pDGCR8-sh. Interestingly, 72 h after transfection with phrGFP and pDGCR8-sh, most of the GFP-positive transfected cells had a clear reduction in the number and size of GWBs (Fig 4vi) compared with cells transfected with phrGFP alone (Fig 4iii). Similar results were observed using pDGCR8-sh2. Fig 4vii-ix shows the reappearance of GWBs in GWB-deficient cells transiently transfected with pDGCR8-sh and subsequently with lamin A/C siRNA (compare Fig 4ix with vi).

Fig 5 is a schematic summary of our interpretation of the pathways involved in mRNA targeting by miRNA and their eventual GWB destination. Some of the steps required for processing of pri-miRNA to mature miRNA or siRNA are well

documented and reviewed elsewhere (Tang, 2005). On the basis of recently published reports (Liu *et al*, 2005a,b; Valencia-Sanchez *et al*, 2006) and on the data presented here, we propose that the initiation of the events leading to the formation of GWBs requires miRNA. We cannot rule out the possibility that miRNA depletion has a direct or indirect effect on the expression of other components required for the assembly of GWBs. However, the ability of siRNA to act as a surrogate for miRNA in restoring GWBs and maintaining RNAi activity argues against this possibility. We further propose that after the initial partnering of the correct mRNA with the Ago2-miRNA complex, and perhaps with the assistance of mRNA-binding proteins such as GW182, more proteins are recruited, leading to the formation of these cytoplasmic foci. The recruited proteins probably include a complex of mRNA degradation-associated proteins (Andrei *et al*, 2005; Fenger-Gron *et al*, 2005; Yu *et al*, 2005). Our data are consistent with previous work showing that actinomycin D, an inhibitor of Pol II, leads to disassembly of GW/P bodies (Cougot *et al*, 2004). It is known that miRNAs are transcribed by Pol II (Lee *et al*, 2004) and therefore inhibition of transcription could lead to the disassembly of GWBs, in part, owing to the lack of miRNA production. In conclusion, GWB formation seems to be miRNA dependent, or most probably Ago2-miRNA and/or

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

METHODS

Short hairpin RNA construct. The short hairpin constructs to Drosha, DGCR8 and Narf were obtained from Open Biosystems (Huntsville, AL, USA) and named pDrosha-sh (clone ID V2HS_71783), pDGCR8-sh (V2HS_98193), pDGCR8-sh2 (V2HS_98477) and pNarf-sh (V2HS_50154). Ten unrelated clones were also purchased and shown to behave similarly to the negative control pNarf-sh.

Cell culture and transfection. HeLa CCL2 cells obtained from ATCC were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HeLa cells seeded to approximately 90% confluency in a six-well plate or eight-chamber slide were transfected with the pDrosha-sh plasmid using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) or Arrest-In (OpenBiosystems, Huntsville, AL, USA). For transient transfections, pDrosha-sh was transfected with phrGFP, maintaining the total DNA amount as per the manufacturer's instructions. To generate a homogeneous population of Drosha-deficient cells, selection pressure was applied using 2 µg/ml puromycin 2 days after the initial transfection of pDrosha-sh. At 10–21 days after the initial transfection, sufficiently large colonies were maintained in puromycin for the preparation of cell lysates suitable for reverse transcription-PCR (RT-PCR) and western blot analysis. Fluorescently labelled miRNA/siRNA was transfected into HeLa cells or Drosha-deficient cells using Oligofectamine (Invitrogen), as recommended. After 48 h of transfection of miRNA/siRNA, samples were processed by IIF, RT-PCR or western blot at 24 or 48 h after transfection.

Other methods. Other material and methods are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank Dr J. Lykke-Andersen, University of Colorado, for providing the rabbit Dcp1 antibody and Dr T. Hobman, University of Alberta, for providing the Ago2 rabbit antibody. This work was supported in part by the Canadian Institutes for Health Research grant MOP-38034, Canadian Breast Cancer Research Foundation grant 16992 and National Institutes of Health grants AI47859 and AR42455. M.J.F. holds the Arthritis Society Chair.

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