Sjögren Syndrome Antigen B (SSB)/La Promotes Global MicroRNA Expression by Binding MicroRNA Precursors through Stem-Loop Recognition*

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Background: MicroRNA biogenesis is a multistep process regulated by RNA-binding proteins.

Results: Sjögren syndrome antigen B (SSB)/La binds stem-loop precursor microRNAs (pre-miRNA) and is required for microRNA expression.

Conclusion: La/SSB promotes global microRNA expression by stabilizing pre-miRNAs from nuclease-mediated decay.

Significance: This study reveals a novel concept of pre-miRNA holding complex that protects and escorts pre-miRNAs in microRNA biogenesis pathway.

MicroRNAs (miRNA) control numerous physiological and pathological processes. Typically, the primary miRNA (pri-miRNA) transcripts are processed by nuclear Drosha complex into ~70-nucleotide stem-loop precursor miRNAs (pre-miRNA), which are further cleaved by cytoplasmic Dicer complex into ~21-nucleotide mature miRNAs. However, it is unclear how nascent pre-miRNAs are protected from ribonucleases, such as MCTP1, that degrade pre-miRNAs to abort microRNA production. Here, we identify Sjögren syndrome antigen B (SSB)/La as a pre-miRNA-binding protein that regulates microRNA processing in vitro. All three RNA-binding motifs (LAM, RRM1, and RRM2) of La/SSB are required for efficient pre-miRNA binding. Intriguingly, La/SSB recognizes the characteristic stem-loop structure of pre-miRNAs, of which the majority lack a 3’ UUU terminus. Moreover, La/SSB associates with endogenous pri-/pre-miRNAs and promotes miRNA biogenesis by stabilizing pre-miRNAs from nuclease (e.g., MCTP1)-mediated decay in mammalian cells. Accordingly, we observed positive correlations between the expression status of La/SSB and Dicer in human cancer transcriptome and prognosis. These studies identify an important function of La/SSB as a global regulator of miRNA expression, and implicate stem-loop recognition as a major mechanism that mediates association between La/SSB and diverse RNA molecules.

MicroRNAs (miRNAs)3 are ~21-nucleotide (nt) cellular RNAs that govern numerous biological and disease processes by directing degradation and translational repression of cognate mRNAs (1–3). It is estimated that human genome encodes up to one thousand miRNAs (1, 3, 4), which are transcribed either as independent genes, or imbedded in other genes. The majority of miRNAs are generated by sequential processing by two RNase III enzymes: Drosha and Dicer. In the nucleus, the primary miRNA (pri-miRNA) transcripts are processed by the Drosha-DGCR8/Pasha complex into ~70-nt stem-loop precursor miRNAs (pre-miRNAs) (5–8). The pre-miRNAs are exported by Exportin 5-Ran-GTP to the cytoplasm (9, 10), and are further cleaved into mature miRNAs by the Dicer-1-Losq-PB complex in Drosohila melanogaster (11–13), or by the Dicer-TRBP complex in mammals (14–17). Both DGCR8/Pasha or TRBP/Losq-PB are dsRNA-binding proteins that promote miRNA processing by stabilizing Drosha and Dicer and by facilitating their interaction with pri-miRNAs and pre-miRNAs, respectively (18–20). A number of post-transcriptional regulators of miRNA biogenesis have recently been characterized, including Lin28, KSRP, hnRNP A1, and SMAD (21–27). All of these miRNA regulators modulate the production of a small subset of miRNAs through recognition of specific sequences of pri- and/or pre-miRNAs (22, 23, 28).

In many RNA maturation processes, the precursor molecules are often bound by RNA-binding proteins or chaperones to protect them from nuclease-mediated decay and to ensure the correct processing by specific ribonucleases. In the current model, however, newly synthesized pri-/pre-miRNAs are

3 The abbreviations used are: miRNA, microRNA; SSB, Sjögren syndrome antigen B; MCTP1, monocyte chemotactic protein-induced protein 1; nt, nucleotide(s); LAM, La motif; RRM, RNA recognition motif; qPCR, quantitative PCR; GSEA, gene set enrichment analysis; IP, immunoprecipitate; RNP, ribonucleoprotein.
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TABLE 1
siRNA targets, primer sequences, and Northern probes

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>siLa1 (human) target sequence</th>
<th>siLa2 (human) target sequence</th>
<th>Cyclophilin (human) forward</th>
<th>Cyclophilin (human) reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-GGUGCCUGUGAGAAGUUGAAGC-3'</td>
<td>5'-GGUGAGUGAUUAGAAAGGAU-3'</td>
<td>5'-UCGCGTTCACCGAGAAG-3'</td>
<td>5'-UCGCGTTCACCGAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCCAGAGGTTGACACTTAA-3'</td>
<td>5'-CTCATACCCACAGGAAACGGA-3'</td>
<td>5'-CTCATACCCACAGGAAACGGA-3'</td>
<td>5'-CTCATACCCACAGGAAACGGA-3'</td>
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...described as unprotected and delivered directly to Drosha or Dicer enzymes for processing. It has recently been suggested that the processing of pri-miRNAs by Drosha occurs co-transcriptionally on chromatin (29, 30). In contrast, nascent pre-miRNAs embark a long and perilous journey from the nucleus to the cytoplasm, in which they are vulnerable to degradation by many nucleases. For example, the monocyte chemotactic protein-induced protein 1 (MCPIP1)/ZC3H12A, a ribonuclease and deubiquitinase that suppresses responses in smokers (31, 32), was recently reported to antagonize Dicer by degrading pre-miRNAs to abort miRNA production (33).

Sjögren syndrome antigen B (SSB), also known as autoimmune antigen La, is commonly associated with autoimmune disorders such as Sjögren syndrome and systemic lupus erythematosus (34). The La/SSB protein is evolutionarily conserved from yeast to human, exists abundantly in both the nucleus and cytoplasm, and plays fundamental roles in diverse processes of RNA metabolism, and possibly the pathogenesis of cancer and autoimmune diseases.

**EXPERIMENTAL PROCEDURES**

**General Reagents and Antibodies**—General RNA reagents were purchased from Ambion and Promega, and DNA oligos or siRNAs were synthesized by IDT. Both Dicer and human La/SSB antibodies were purchased from Santa Cruz Biotechnology, whereas actin, tubulin, and anti-FLAG antibodies were obtained from Sigma. Recombinant *Drosophila* Dicer-1, Loqs-PB and human Dicer*TRBP* complexes were generated in insect cells as previously described (12, 17). Recombinant *Drosophila* and human La/SSB were produced in *Escherichia coli* and purified by nickel affinity and ion-exchange chromatography. Recombinant FLAG-tagged MCPIP1 and Drosha-DGCR8 complex was affinity purified from 293T cells following transient transfection (47).

**Purification of La/SSB from S2 Extract**—The cytoplasmic extract (S100) of *Drosophila* S2 cells (48) was precipitated by ammonium sulfate at 60% saturation. After a 30-min, 20,000 × g centrifugation step, the supernatant was dialyzed overnight in Buffer A (10 mM KOAc, 10 mM HEPES, pH 7.4, 2 mM Mg(OAc)₂, 2.5 mM DTT), loaded onto a Mono S column, and eluted with a 300–600 mM NaCl gradient. The fractions with peak activity were dialyzed for 4 h, loaded onto a Mono Q column, and eluted with a 0–300 mM NaCl gradient. Finally, the...
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peak fractions were directly loaded on a Smart Mono S column and eluted with a 300–600 mM NaCl gradient.

Immunoprecipitation of La/SSB Complexes—HeLa cell extract was prepared in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA) freshly supplemented with protease inhibitors. HeLa extract was incubated with anti-human La/SSB monoclonal antibody or mouse IgG for 1 h at 4 °C, and protein G-Sepharose beads were added and rotated for another 4 h. After extensive wash of the beads (4 times in RIPA buffer, 3 times in RIPA buffer with 0.5 M NaCl, and once with buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0)), associated RNA was extracted from the beads by TRIzol and precipitated by ethanol.

Cross-linking and Immunoprecipitation Assay—To demonstrate in vivo association between La and pre-miRNAs, we transfected HeLa cells with a empty vector or FLAG-hLa/SSB expression construct. Thirty-six hours after transfection, HeLa cells were either untreated or treated with 1.0% formaldehyde before making protein extracts in RIPA buffer (49). The FLAG-La RNP complexes were immunoprecipitated from HeLa cell extracts by incubation with anti-human La/SSB monoclonal antibody or mouse IgG for 1 h at 4 °C, and protein G-Sepharose beads were added and rotated for another 4 h. After extensive wash of the beads (4 times in RIPA buffer, 3 times in RIPA buffer with 0.5 M NaCl, and once with buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.0)), associated RNA was extracted from the beads by TRIzol and precipitated by ethanol.

Quantitative Analysis of miRNA Expression—Northern blotting for pre-miRNA and miRNA detection was performed essentially as described (50). Typically, 30 μg of total RNA was resolved by 12% urea-PAGE and transferred to nylon membrane. Hybridization was conducted in ultrasonicated hybridization buffer (Ambion) at 40 °C overnight. The membrane was washed 3 times at 40 °C in 2× SSC, 0.5% SDS, and exposed to x-ray film.

FIGURE 1. Purification and reconstitution of La/SSB as a regulator of pre-miRNA processing. A, Coomassie-stained SDS-PAGE showing recombinant Drosophila Dicer-1 Loqs-PB complex purified from insect cell extracts. B, the pre-miRNA processing assay was performed in buffer (lane 1) or with 10 or 30 ng of recombinant Dicer-1 Loqs-PB complex in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 60% ammonium sulfate supernatant (60S) of S2 extract. C, purification of endogenous La/SSB from S2 extract by sequential chromatographic fractionation. At the final Mono S step, individual fractions were assayed for pre-miRNA processing activity (top) or resolved by SDS-PAGE followed by silver staining (bottom). D, Coomassie-stained SDS-PAGE showing purified Drosophila (dLa) or human (hLa) La/SSB. E, the pre-miRNA processing assays were performed using ~10 nm recombinant Dicer-1 Loqs-PB complex with increasing concentrations of recombinant dLa. F, the pre-miRNA processing assays were conducted using ~10 nm recombinant Dicer-TRBP complex with increasing concentrations of recombinant hLa. G, the pre-miRNA processing assays were conducted using 10 μg of crude S2 extract with increasing concentrations of recombinant dLa. H, the pre-miRNA processing assays were conducted using 10 μg of HeLa extract with increasing concentrations of recombinant hLa.
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Global miRNA Profiling—For RT-PCR, cDNAs were generated using the reverse transcription kit (Applied Biosystems) and amplified by PCR with Taq polymerase. The primer sequences are listed in Table 1. TaqMan qPCR was performed to measure the levels of specific miRNAs or pri-miRNAs according to the manufacturer’s protocol (Applied Biosystems). Relative quantities were calculated using the \( \Delta\Delta C_t \) method. 18S rRNA served as a loading control. Global miRNA profiling was performed using MegaPlex RT Primers and TaqMan Array microRNA Cards (Applied Biosystems, Human Pool A version 2.1 MegaPlex RT primers and Human MicroRNA A Cards version 2.0) according to the manufacturer’s protocols. 750 ng of total RNA was used in Megaplex RT systems. Relative quantities were calculated using the \( C_t \) values among replicates and a \( p \) value threshold of \( p \leq 0.05 \).

Native Gel-shift Assays—Synthetic pre-miRNAs were 5’ radiolabeled with \([\gamma-32P]ATP\) by T4 polynucleotide kinase followed by G-25 column (Ambion) purification. The pri-let-7a-1 construct, a gift from Dr. Narry Kim, was used to generate uniformly radiolabeled pri-miRNA substrate by in vitro transcription followed by PAGE purification. Typically, recombinant proteins and radiolabeled RNA were incubated at 37°C (human proteins) or 30°C (Drosophila proteins) for 30 min in a 10-μl reaction (100 mM KOAc, 15 mM HEPES, pH 7.4, 2.5 mM EDTA, 2.5 mM DTT, pH 7.4). The reaction mixture was resolved by a 5% native PAGE and exposed to x-ray film.

Gene Set Enrichment Analysis (GSEA)—Gene expression profiling data of breast cancer patients were obtained from the NCBI Gene Expression Omnibus (GSE7390) and bioinformatics.ncbi.nlm.nih.gov/data.php. The expression levels of Dicer, La/SSB, or MCPIP1 were evaluated by the corresponding probes. Genes negatively or positively associated with Dicer expression (“Dicer high/low down-regulated or up-regulated genes”) were defined as 200 of the most differentially expressed genes between the top 30 cases with high and low Dicer expression. After dividing all cases into halves according to La expression, GSEA was performed with GSEA software available from the Broad Institute (51). Survival analysis was performed using the survival package of R, the survfit function, and the survdiff step, only a single protein of ∼50 kDa appeared on the silver-stained polyacrylamide gel (PAGE), and correlated closely with the miRNA regulatory activity (Fig. 1C). This protein was identified as the Drosophila homolog of La/SSB by mass spectrometric analysis.

We generated His-tagged Drosophila dLa and human hLa La/SSB recombinant proteins (Fig. 1D). Addition of dLa efficiently suppressed the ability of the recombinant Dicer-1-Loqs-PB complex to process pre-miRNA into miRNA in vitro (Fig. 1E). Likewise, hLa could inhibit pre-miRNA processing by the recombinant Dicer-TRBP complex (Fig. 1F). Intriguingly, we obtained a strikingly different result when performing pre-miRNA processing assays using crude cell extracts. Addition of

RESULTS

Purification and Reconstitution of La/SSB as a Regulator of Pre-miRNA Processing in Vitro—We took an unbiased biochemical approach to identify new regulators of miRNA biogenesis by supplementing recombinant Dicer-1-Loqs-PB complex (Fig. 1A) with the fractions of cytoplasmic extract (S100) of Drosophila S2 cells. We observed an inhibitory activity of pre-miRNA processing in the supernatant after 60% saturation of ammonium sulfate precipitation of S2/S100 (Fig. 1B). This factor was further purified to homogeneity by sequential chromatographic fractionation (Fig. 1C). At the final purification step, only a single protein of ∼50 kDa appeared on the silver-stained polyacrylamide gel (PAGE), and correlated closely with the miRNA regulatory activity (Fig. 1C). This protein was identified as the Drosophila homolog of La/SSB by mass spectrometric analysis.

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La/SSB could instead stabilize pre-miRNA and enhance miRNA production in both S2 and HeLa cell extracts (Fig. 1, G and H). The degree of miRNA increase was smaller than that of the pre-miRNA increase, suggesting a combined effect of pre-miRNA stabilization and sequestration at work.

La/SSB Is a Pre-miRNA-binding Protein—Recombinant dLa could efficiently bind pre-miRNA and compete with Dicer-1-Loqs-PB complex for pre-miRNA binding in vitro (Fig. 2A), suggesting that the binding of pre-miRNA by dLa is incompatible with that of the Dicer complex. Furthermore, recombinant dLa and Dicer-1-Loqs-PB exhibited comparable binding affinity for pre-miRNA in vitro (Fig. 2, B and C). A similar phenomenon was observed for recombinant hLa and Dicer-TRBP complex (data not shown). These results suggest that pre-miRNAs could be readily exchanged between La/SSB and the Dicer complex. Therefore, in a recombinant system, La/SSB could suppress miRNA production by sequestering pre-miRNA away from Dicer. However, in the crude extract or live cells, we hypothesized that La/SSB might promote miRNA expression by protecting pre-miRNAs from nuclease-mediated decay to allow for productive processing by Dicer.

The LAM, RRM1, and RRM2 Motifs of La/SSB Are All Required for Efficient Pre-miRNA Binding—To map which domains of La/SSB are important for binding pre-miRNA, we generated a series of truncated human La/SSB recombinant proteins (Fig. 3, A and B), and compared their pre-miRNA binding by gel-shift assays. Although LAM-RRM1 showed weak binding to pre-miRNA, neither LAM nor RRM1-RRM2 was able to bind pre-miRNA (Fig. 3C). In contrast, LAM-RRM1-RRM2 could bind pre-miRNA almost as efficiently as full-length La/SSB (Fig. 3C). Thus, the LAM, RRM1, and RRM2 motifs of La/SSB are all required for efficient binding of pre-miRNA. This mode of interaction is clearly distinct from the previously established mode of 3' UUU recognition by LAM-RRM1 (40–42).

La/SSB Regulates Pre-miRNA Processing by Binding Pre-miRNA—Two tyrosines (Tyr-23, Tyr-24) in the LAM of human La/SSB have previously been shown to be critical for 3' UUU binding (34). In our assays, mutations of Tyr-23 and Tyr-24 to alanine (YY) diminished the ability hLa to bind pre-miRNA (Fig. 3, D and E). Accordingly, the YY mutant also lost its ability to either inhibit pre-miRNA processing by recombinant Dicer-1-Loqs-PB complex (Fig. 3F), or stabilize pre-miRNA and enhance miRNA production in HeLa extract (Fig. 3G). These results suggest that the activity of La/SSB to regulate pre-miRNA processing is dependent on its ability to bind pre-miRNA.

La/SSB Binds Pre-miRNAs via a 3' UUU-independent Mechanism—Profiling of the miRNA database found that the majority of Drosophila and human pre-miRNAs lack the 3' UUU terminus (Fig. 4A). Among the 240 fly pre-miRNAs, only three have 3 UUs out of the last four nucleotides. Among the 1,527 human pre-miRNAs, only six carry a 3' UUUU tail and 21 have 3 Us within the last four nucleotides. Accordingly, recombinant hLa could efficiently bind a number of synthetic pre-miRNAs lacking 3' UUU (data not shown). Moreover, we carefully compared the binding of hLa to mouse pre-miR-23b (lacking 3' UUU) and a modified pre-miR-23b that carried 3' UUU (Fig. 4, B and C). These experiments showed that addition of 3' UUU made little or no change to the binding of La/SSB to pre-miR-23b. Taken together, these studies suggest that La/SSB primarily binds pre-miRNAs via a 3' UUU-independent mechanism.

La/SSB Recognizes the Stem-Loop Structure of Pre-miRNAs—Because all pre-miRNAs share a stem-loop structure, we hypothesized that La/SSB could recognize the common stem-loop structure rather than specific sequences of pre-miRNAs. To test this idea, we synthesized a series of truncated forms of human pre-let-7a-1, which consists of a 21-bp imperfect double-stranded stem and a 27-nt single-stranded loop (21–27)
Shortening the loop of pre-let-7a-1 from 27 to 4 nt reduced the binding of hLa, whereas deleting the loop abolished the binding of hLa to pre-let-7a-1, as in the case of let-7a/let* miRNA duplex (Fig. 5, B and D). Thus, the loop of pre-miRNA is critical for La/SSB binding, but in a sequence-independent manner as the binding of pre-let-7a-1 was not affected by substituting its loop with antisense sequence (data not shown). However, the La/SSB-pre-miRNA interaction cannot be explained by the loop binding alone because the binding of pre-let-7a-1 could not be competed off with an excess amount of ssRNA (data not shown).

Surprisingly, shortening the double-stranded stem of pre-let-7a-1 had little impact on the binding of hLa, whereas deleting the loop abolished the binding of hLa to pre-let-7a-1, as in the case of let-7a/let* miRNA duplex (Fig. 5, B and D). Thus, the loop of pre-miRNA is critical for La/SSB binding, but in a sequence-independent manner as the binding of pre-let-7a-1 was not affected by substituting its loop with antisense sequence (data not shown). However, the La/SSB-pre-miRNA interaction cannot be explained by the loop binding alone because the binding of pre-let-7a-1 could not be competed off with an excess amount of ssRNA (data not shown).

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tion between endogenous La/SSB and pri-miRNAs was further confirmed by Northern blotting (Fig. 6C). To eliminate the possibility that La/SSB only associated with pri-miRNAs after cell lysis, we demonstrated in vivo association between FLAG-La and pri-miRNAs by cross-linking of live HeLa cells followed by co-IPs under stringent washing conditions that only tolerated covalent RNA-protein linkage (Fig. 6D) (49).

**La/SSB Associates with Pri-miRNAs in Vitro and in Vivo**—
We also examined the association of endogenous La/SSB and pri-miRNA transcripts in the nuclear extract of HeLa cells. The presence of pri-miRNAs, but not cyclophilin pre-messenger RNA, was specifically detected by reverse transcription (RT)-PCR in the La/SSB RNP complexes (Fig. 6E). Furthermore, recombinant hLa could directly bind radiolabeled pri-miRNA in vitro as shown by native gel-shift assay (Fig. 6F). Interestingly, the binding of hLa to pri-miRNA appeared compatible with that of the Drosha-DGCR8 complex (Fig. 6F, compare lanes 1–4). This was confirmed by the supershift of the complex of hLa, Drosha-DGCR8, and pri-miRNA caused by anti-hLa/SSB antibodies (Fig. 6F, compare lanes 7 and 8 to lanes 3, 4, and 6). Accordingly, addition of hLa had little effect on in vitro pri-miRNA processing by recombinant Drosha-DGCR8 complex (data not shown). Collectively, these studies indicate that La/SSB associates with cellular pri-/pre-miRNAs in vivo.

**La/SSB Is Required for Global miRNA Expression in Vivo**—
To determine whether La/SSB regulates miRNA expression in vivo, we performed siRNA-mediated knockdown of La/SSB expression in HeLa cells (Fig. 7A), and followed with Northern blotting to measure the levels of six endogenous miRNAs. Consistent with our hypothesis that La/SSB promotes miRNA expression by stabilizing pre-miRNAs, there was a substantial reduction of all six miRNAs in La/SSB-depleted cells as compared with the control siRNA-treated cells (Fig. 7B). In contrast, the levels of 5.8S or 5S rRNA and U6 RNA remained unchanged (Fig. 7B), suggesting that the lack of La/SSB had a specific impact on miRNA expression.

Furthermore, we examined the global effect of La/SSB knockdown on miRNAs by profiling the expression of 377 mature miRNAs by TaqMan qPCR in control and La/SSB siRNA-treated HeLa cells (Fig. 7C). Approximately 58% (94 of 161) of the expressed miRNAs displayed a statistically significant ($p < 0.05$) reduction in the La/SSB-deficient cells, whereas U6 RNA expression was unchanged. The remainder of expressed miRNAs displayed either low ($C_t > 32$) or variable ($p > 0.05$) expression. None of the miRNAs exhibited increased expression.
in repeated experiments. These findings were further validated by individual TaqMan qPCR of randomly selected miRNAs (data not shown). These results suggest that La/SSB is required for global miRNA expression in vivo.

La/SSB Promotes miRNA Expression by Stabilizing Pre-miRNAs—We compared the levels of pri-, pre-, and mature miRNAs between control and La/SSB-depleted HeLa cells by TaqMan qPCR and Northern blotting. Although the levels of
some pri-miRNAs remained the same, others were slightly decreased upon La/SSB depletion (Fig. 8A). In general, we detected a more substantial reduction of pre-miRNAs that was consistent with the degree of miRNA reduction in La/SSB-deficient cells (Fig. 8, A and B, and data not shown). Notably, although the level of pri-miR-23b remained unchanged, both pre-miR-23b and mature miR-23b were reduced by ~30% when La/SSB was depleted (Fig. 8, A and B). A similar phenomenon was observed in Tet on-inducible La/SSB knockdown osteosarcoma (U2OS) cells. In these cells, induction of a short hairpin RNA (shLa) could effectively silence endogenous La/SSB expression, resulting in the concomitant reduction of pre-miRNAs and miRNAs (Fig. 8C). In another set of experiments, we also knocked down the expression of Dicer to allow for accumulation of pre-miRNAs in U2OS/shLa cells. In this case, we observed a much more dramatic reduction of pre-miRNAs when both La/SSB and Dicer were depleted (Fig. 8, D and E). Collectively, these results suggest that La/SSB promotes miRNA expression by stabilizing pre-miRNAs in mammalian cells.

**La/SSB Protects Pre-miRNA from MCPIP1-mediated Degradation**—In further support of this idea, we found that radio-labeled pre-miRNA was much less stable in the La/SSB-depleted extract than in the control extract (Fig. 9A). Thus, the lack of La/SSB likely rendered pre-miRNAs more vulnerable to degradation by ribonucleases other than Dicer. The first example of such ribonucleases is MCPIP1, which was recently identified to inhibit miRNA production by cleaving the loop region of specific pre-miRNAs (33). Here, we showed that addition of hLa protected pre-miRNA from recombinant MCPIP1-mediated degradation \textit{in vitro} (Fig. 9B). Furthermore, co-expression of hLa could antagonize the negative effect of MCPIP1 overexpression by stabilizing pre-miRNAs to enhance miRNA expression (Fig. 9C). Finally, the levels of both pre-miRNAs and miRNAs could be restored in La/SSB-deficient U2OS/shLa cells by expression of a FLAG-tagged \textit{Drosophila} La/SSB transgene (Fig. 9, D–F). This experiment not only confirms that the miRNA phenotype is specifically due to the La/SSB deficiency, but also suggests that La/SSB plays an evolutionarily conserved role in miRNA regulation from flies to human. Therefore, our genetic and biochemical studies demonstrate that La/SSB promotes global miRNA expression by stabilizing pre-miRNAs from nuclease (e.g. MCPIP1)-mediated degradation.

**Cooperative Relationships between La/SSB and Dicer in Human Cancers**—It was previously reported that MCPIP1 and Dicer are in a antagonistic relationship in the transcriptome.
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FIGURE 8. La/SSB promotes miRNA expression by stabilizing pre-miRNAs. A, quantitative analysis of pri-miRNA expression between siGFP- and siLa-treated HeLa cells by TaqMan PCR. 18S rRNA was used as a loading control. B, quantitative analysis of pre-miRNA and miRNA expression between siGFP- and siLa-treated HeLa cells by Northern blotting. 5.8S rRNA was used as loading control. C, Northern analysis of pre-miR-16 and miR-16 expression in two U2OS/shLa cell lines cultured in the absence or presence of 2 μM doxycycline. Western blotting was performed to compare the levels of La/SSB or actin protein. D, Northern analysis of pre-miR-21 and miR-21 expression in U2OS/shLa cells, in which La/SSB, Dicer, or both were knocked down by RNAi. Western blotting was performed to compare the levels of La/SSB, Dicer, or actin protein. E, Northern analysis of the levels of various pre-miRNAs in U2OS/shLa cells, in which expression of La/SSB and/or Dicer was knocked down.

FIGURE 9. La/SSB protects pre-miRNAs from nuclease (e.g. MCPIP1)-mediated decay. A, a time course experiment comparing stability of a 5′ radiolabeled pre-miRNA in La− versus La+ extract prepared from U2OS/shLa cells cultured without (La−) or with doxycycline (La+). B, in vitro RNase assay showing that addition of hLa protects 5′ radiolabeled pre-miRNA from recombinant MCPIP1-mediated degradation. C, Northern analysis of pre-miR-16 and miR-16 expression in 293T cells transiently transfected with a pri-miR-16 expression construct and vector, FLAG-MCPIP1 and/or FLAG-hLa constructs. D, Western blotting was performed to detect endogenous hLa, transgenic FLAG-dLa, and actin in U2OS/shLa or U2OS/shLa (FLAG-dLa) cells. E, Northern analysis of pre-miR-21 and miR-21 expression in U2OS/shLa or U2OS/shLa (FLAG-dLa) cells cultured without or with doxycycline. F, quantitative analysis of the data in E for four independent sets of experiments.
data of lung cancer patients (33). Thus, we analyzed the functional relationship between Dicer and La/SSB in human malignancies. To this end, we performed GSEA (51) to examine whether genes associated negatively or positively with Dicer expression status are overexpressed or underexpressed along with La/SSB expression status, using several public gene expression datasets of human malignancies. Intriguingly, we observed a potential cooperative relationship between Dicer and La/SSB in human breast cancer datasets (53). In this dataset, the Dicer high/low down-regulated genes are enriched in patients with low La/SSB expression (“La low case”) (Fig. 10A). Conversely, the Dicer high/low up-regulated genes are enriched in patients with high La/SSB expression (“La high case”) (Fig. 10A). This cooperative relationship between Dicer and La/SSB is opposite to the antagonistic relationship between Dicer and MCPIP1 in human cancer transcriptome (33).

Furthermore, low levels of La/SSB and Dicer were both associated with poor clinical outcome in breast cancer patients (Fig. 10B). Conversely, a high level of MCPIP1 is associated with worse prognosis. Although we failed to detect statistical significance in the latter case, the general trend is similar to what was previously reported in lung cancer patients (33). We also observed similar correlations in another dataset of breast cancer patients (54) (data not shown). Thus, these results highlight an interesting tripartite relationship among Dicer, La/SSB, and MCPIP1 in the context of human malignancies. Taken together, these studies suggest that consistent with their critical roles in miRNA expression, the expression status of Dicer and La/SSB might have cooperative effects on the transcriptome and prognosis of certain human cancers.

DISCUSSION

In summary, we have identified a new housekeeping function of autoantigen La/SSB in promoting global miRNA expression by binding and stabilizing pre-miRNAs. We propose a revised working model for the basic process miRNA biogenesis. In the nucleus, La/SSB associates with nascent pri-miRNA transcripts and is required for the stability of some pri-miRNAs. The binding of La/SSB to pri-miRNAs is compatible with binding of the Drosha complex that processes pri-miRNAs into pre-miRNAs. Nascent pre-miRNAs are protected by La/SSB from degradation by other nucleases, such as MCPIP1. The comparable binding affinity allows pre-miRNAs to be easily transferred from La/SSB to the Dicer complex for processing into mature miRNAs. It is also plausible that this transfer process may be
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influenced by other proteins or by post-translational modifications. The work reveals a new concept of the pre-miRNA holding complex that protects and escorts nascent pre-miRNAs through the miRNA biogenesis pathway.

The La/SSB protein is well known for its versatile interactions with diverse RNA molecules, including numerous Pol III transcripts, specific cytoplasmic mRNA, and viral-encoded transcripts (35). Most of previous studies have focused on the interaction between La/SSB and 3′ UUU of Pol III transcripts, such as pre-tRNAs. However, a few recent studies have reported that La/SSB associate with specific cellular and viral transcripts through a poorly understood, 3′ UUU-independent mechanism (35). La/SSB has recently been shown to bind the domain IV stem-loop sequence of the internal ribosome entry site (IRES) of hepatitis C virus mRNA (66). La/SSB recognizes the structure rather than specific sequence of domain IV RNA, and this association requires its three RNA binding motifs (i.e. LAM, RRM1, and RRM2). Herein, we have determined that La/SSB recognizes the typical stem-loop structure of pre-miRNAs. These studies implicate that stem-loop recognition may serve as a new mechanism that mediates association between La/SSB with diverse RNA lacking 3′ UUU.

It is possible that additional sequence features may further modulate the binding of La/SSB to pre-miRNAs. Lin28 recruits terminal uridylintransferase TUT4 to the polyuridylate 3′ end of a small subset of pre-miRNAs, including pre-let-7 (26, 28). In this case, however, 3′ polyuridylation (addition of 5 to 19 Us) of pre-miRNA inhibits Dicer processing and leads to premature pre-miRNA degradation. It is unclear how many endogenous pre-miRNAs are uridylated at the 3′ terminus. Our studies indicate that addition of 3′ UUU to a pre-miRNA have little effect on La/SSB binding. Thus, the interaction between La/SSB and pre-miRNA is primarily mediated by stem-loop recognition. This mode of interaction is similar to the mechanism of how the Dicer complex recognizes pre-miRNAs of diverse sequences.

Although La/SSB is dispensable for yeast viability (43, 44), La/SSB-deficient flies and knock-out mice die at early embryonic stage and La/SSB+/− blastocysts fail to develop into embryonic stem cells in vitro (45, 46). These phenotypes are reminiscent of those displayed by mutant flies or mice lacking key miRNA-generating factors, such as Drosha, Dicer, Loqs, or Pasha/DGCR8 (11, 55–59). Because miRNAs do not exist in the budding or fission yeast, these observations are consistent with our theory that La/SSB plays an essential role in higher eukaryotes through regulation of global miRNA expression.

The previous and current studies suggest a dynamic process where La/SSB, MCPIP1, and Dicer compete to regulate miRNA production by modulating the levels of pre-miRNAs (33). Alterations of miRNA expression are linked to a wide range of pathological processes, including cancer (1, 3). It has been shown that the miRNA machineries can be modified by multiple mechanisms in human cancers. Such aberrant regulations include down-regulation of the miRNA processing enzymes, e.g., Dicer and Drosha (52, 60), and mutations of cofactors, e.g., TRBP, Exportin-5 (63, 64), and possibly La/SSB (this study). In several types of human malignancies, there are clear correlations between impaired miRNA processing, e.g., low expression of Drosha, Dicer, and La/SSB or high expression of MCPIP1, and poor prognosis of cancer patients (33, 60–62). Collectively, these observations strongly suggest that a global reduction in miRNA expression, coupled with overexpression of specific oncopgenic miRNAs, may be a distinct feature for certain, if not all, of human cancers.

Mounting studies indicate that miRNAs play critical roles in maintaining the homeostasis of the immune system and safeguarding autoimmunity (63). Human patients suffering Sjögren syndrome and systemic lupus erythematosus frequently produce autoantibodies against specific classes of RNA-binding proteins. These autoantigens include the commonly known La/SSB and Ro, as well as components of the miRNA pathway, such as Dicer, Argonaute, and GW182 (64, 65). Here, we have identified that autoantigen La/SSB also functions as a key regulator of global miRNA expression. Therefore, it is imperative to investigate if inactivation of La/SSB, such as by autoantibodies, down-regulation of expression, and/or phosphorylation, contributes directly to the pathogenesis of these autoimmune diseases.

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REFERENCES

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