Inositol phosphatase SHIP1 is a primary target of miR-155

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MicroRNA-155 (miR-155) has emerged as a critical regulator of immune cell development, function, and disease. However, the mechanistic basis for its impact on the hematopoietic system remains largely unresolved. Because miRNAs function by repressing specific mRNAs through direct 3’UTR interactions, we have searched for targets of miR-155 implicated in the regulation of hematopoiesis. In the present study, we identify Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) as a direct target of miR-155, and, using gain and loss of function approaches, show that miR-155 represses SHIP1 through direct 3’UTR interactions that have been highly conserved throughout evolution. Repression of endogenous SHIP1 by miR-155 occurred following sustained over-expression of miR-155 in hematopoietic cells both in vitro and in vivo, and resulted in increased activation of the kinase Akt during the cellular response to LPS. Furthermore, SHIP1 was also repressed by physiologically regulated miR-155, which was observed in LPS-treated WT versus miR-155−/− primary macrophages. In mice, specific knockdown of SHIP1 in the hematopoietic system following retroviral delivery of a miR-155-formatted siRNA against SHIP1 resulted in a myeloproliferative disorder, with striking similarities to that observed in miR-155-expressing mice. Our study unveils a molecular link between miR-155 and SHIP1 and provides evidence that repression of SHIP1 is an important component of miR-155 biology.

Natural text: In recent years, microRNAs have emerged as critical regulators of gene expression in a variety of mammalian cell types, including cells of the immune system (1–3). Through their ability to repress expression of specific target genes via direct 3’UTR interactions, several miRNAs have been shown to impact both physiological and pathological immune processes (4–12). Among the most prominent and well-studied immune system miRNAs to date, microRNA-155 (miR-155) clearly is involved in protective immunity when properly regulated, yet contributes to malignant conditions upon its dysregulated expression.

miR-155 is expressed in a variety of immune cell types, including B cells (8, 9, 13–17), T cells (18), macrophages (19, 20), dendritic cells (8), and progenitor/stem cell populations (7, 21). Interestingly, miR-155 is found at low levels in most of these cell types until their activation by immune stimuli, such as antigen, Toll-like Receptor ligands, and inflammatory cytokines, which rapidly increase miR-155 expression (8, 9, 18–20). Consistent with its expression pattern, miR-155 appears to function in hematopoiesis and the immune response (7–9). For example, defective germinal center formation and antibody isotype class-switching have been observed in miR-155−/− mice following infection or vaccination (8, 9, 22). In T cells, miR-155 promotes skewing toward the Th1 subset (8, 9). In dendritic cells, miR-155 is necessary for proper activation of responder T cells in the context of antigen presentation (8).

Enhanced expression of miR-155 occurs constitutively in a subset of cancer cells of lymphoid (13–17) and myeloid origin (7, 23). We and others have recently demonstrated that sustained expression of miR-155 in the hematopoietic system leads to pathological outcomes. Our group expressed miR-155 ubiquitously in the hematopoietic compartment via bone marrow transfer of hematopoietic stem cells (HSCs) infected with a retroviral vector. This caused a myeloproliferative disorder (MPD) characterized by increased granulocye/monocyte (GM) populations in the bone marrow, peripheral blood, and spleen, impaired erythropoiesis, and severe splenomegaly because of extramedullary hematopoiesis (7). Costinean et al. found that transgenic expression of miR-155 from a B-cell specific promoter can trigger a B-cell malignancy (4).

While much has been learned about miR-155-expression patterns and functions, the molecular basis underlying its biology is relatively uncharacterized. As a result, we have been searching for candidate targets of miR-155 implicated in overlapping biological processes. Using microarray technology, bioinformatics, and an extensive review of the literature, we have identified the inositol phosphatase SHIP1 as a target of miR-155. Through both gain and loss of function approaches, we demonstrate that miR-155 represses SHIP1 through direct 3’UTR interactions during both sustained and physiological expression of miR-155. Furthermore, specific knockdown of SHIP1 in the hematopoietic system using a miR-155-formatted siRNA against SHIP1 largely recapitulated the MPD phenotype we previously described in miR-155-expressing mice. Together, our data demonstrate a molecular link between miR-155 and SHIP1 in the immune system, and suggest that repression of SHIP1 is a critical aspect of miR-155 function.

Results

MicroRNA-155 Represses SHIP1 Through 3’UTR Interactions. We previously performed a mRNA microarray analysis using RAW 264.7 macrophages stably expressing miR-155 to identify possible targets of miR-155 (7). Among the targets was SHIP1 (INPP5D), a gene that is repressed by miR-155 and which has a conserved 8-mer target “seed” in its 3’UTR (Fig. 1A). SHIP1 was of particular interest because miR-155 is the only miRNA with a highly conserved binding site located in the SHIP1 3’UTR according to the TargetScan algorithm (24), and because mice deficient in SHIP1 suffer from a myeloproliferative condition resembling that which we previously described for mice expressing miR-155 (7, 25–28).

To directly test whether miR-155 can repress SHIP1 through direct 3’UTR interactions, we cloned the 3’UTR of SHIP1 into a reporter plasmid downstream from luciferase and performed
reporter assays using 293 T cells. While miR-155 produced from a cotransfected plasmid repressed expression of luciferase fused to the WT SHIP1 3′UTR, it failed to repress the SHIP1 3′UTR containing a mutated miR-155 seed sequence (Fig. 1B). As controls, miR-155 repressed the Picalm 3′UTR and 2-mer control constructs, but not the control UTR without miR-155 sites (see Fig. 1B). These data reveal that miR-155 directly targets the SHIP1 3′UTR leading to repressed expression.

To determine whether miR-155 can repress endogenous SHIP1, we assayed SHIP1 expression in RAW 264.7 cells expressing WT human miR-155, human miR-155 containing a mutated seed region, or vector control. SHIP1 was measured at the mRNA and protein levels by quantitative PCR (qPCR) and Western blotting, respectively. WT miR-155 repressed SHIP1 expression at this time point. Protein levels of SHIP1 were assayed at 0 h, 4 h, and 24 h after LPS treatment of both WT and miR-155 derivatives. Consistent with reduced SHIP1 levels, cells expressing WT human miR-155 exhibited increased activation of Akt following LPS treatment, while Akt activation was similar in the control and human miR-155 seed mutant-expressing cells (Fig. 1G). The different cell types were stimulated with LPS (200 ng/ml) over the indicated time course and Ser-473-phosphorylated-Akt (p-Akt), total Akt, and β-Actin were assayed by Western blotting. Data represent at least 2 independent experiments.

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Enhanced Expression of SHIP1 in miR-155−/− Macrophages Following LPS Treatment. We next evaluated whether SHIP1 is regulated by miR-155 under physiological conditions. To achieve this, we generated bone marrow-derived macrophages (BMMs) from either WT or miR-155−/− mice. As LPS has been shown to be a potent inducer of miR-155 in macrophages (19), we stimulated these cells with LPS over a time course. In WT cells, rapid induction of the miR-155 precursor, BIC, was followed shortly after by elevated expression of mature miR-155 (Fig. 2A and B), as previously seen with poly (I:C)-treated BMMs (19). We also noticed that while BIC levels fall considerably by 24 h, miR-155 expression peaks at this time point. Protein levels of SHIP1 were assayed at 0 h, 4 h, and 24 h after LPS treatment of both WT and miR-155−/− BMMs. We observed no change in SHIP1 protein levels up to 4 h following LPS treatment in cells of both genotypes (Fig. 2C). After 24 h, both WT and miR-155−/− BMMs demonstrated an increase in SHIP1 expression as compared with earlier time points. However, miR-155−/− BMMs had an enhanced level of SHIP1 protein compared to WT control

- **Fig. 1.** MicroRNA-155 represses SHIP1 expression through 3′ UTR interactions. (A) Schematic layout of the SHIP1 mRNA coding sequence (CDS) and 3′ UTR, with the relative location of the miR-155 binding site. Depiction is not to scale. Sequence of mouse and human miR-155 and predicted interaction with conserved 8-mer miR-155 seeds found within the SHIP1 3′UTR from different species (highlighted) are shown. The sequence of the miR-155 seed-mutant vector (Fig. 1C) or vector control (Fig. 1C) was assayed at 0 h, 4 h, and 24 h after LPS treatment of the different RAW 264.7 derivatives. Consistent with reduced SHIP1 levels, cells expressing WT human miR-155 exhibited increased activation of Akt following LPS treatment, while Akt activation was similar in the control and human miR-155 seed mutant-expressing cells (Fig. 1G). The different cell types were stimulated with LPS (200 ng/ml) over the indicated time course and Ser-473-phosphorylated-Akt (p-Akt), total Akt, and β-Actin were assayed by Western blotting. Data represent at least 2 independent experiments.

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versus the WT 0-h sample is shown. Data represent at least 2 independent experiments while qPCR data were normalized to L32. The fold-increase in SHIP1 expression reflected similar differences between WT and miR-155 expressing developing myeloid populations, erythroid precursors, and megakaryocytes compared to control mice (see Fig. 5A). Histological analyses of Wright-stained bone marrow smears confirmed the presence of pathological myeloproliferative conditions in miR-155- and siSHIP1-expressing mice, characterized by elevated numbers of GM progenitors at various stages of development compared to controls (see Fig. 4B). There was also a reduction in developing erythroid precursors and megakaryocytes in both miR-155 and siSHIP1 mice. Of note, miR-155 mice did exhibit a subtle increase in the number of dysplastic granulocytic cells compared with siSHIP1, possibly because of an additional miR-155 target. Flow cytometry also identified that both miR-155- and siSHIP1-expressing cells, which are GFP positive, are responsible for the increased myeloid populations (CD11b+) in the bone marrow (see Fig. 4C).

H&E staining of fixed spleen sections from miR-155 or siSHIP1 mice revealed expanded interfollicular regions containing developing myeloid populations, erythroid precursors, and megakaryocytes compared to control mice (see Fig. 5B). The normal follicular architecture of the spleen was disrupted by

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**Fig. 2.** Enhanced expression of SHIP1 in miR-155-/- macrophages following LPS treatment. Bone marrow-derived macrophages from WT or miR-155-/- mice were stimulated with 10-ng/ml LPS from *Escherichia coli* for the indicated periods of time. Expression of BIC (A) or mature miR-155 (B) was assayed by qPCR. Expression of SHIP1 was assayed in BMMs by Western blotting (C) and qPCR (D). β-Actin was assayed as a loading control for the Western blot, while qPCR data were normalized to L32. The fold-increase in SHIP1 expression versus the WT 0-h sample is shown. Data represent at least 2 independent experiments.

**Fig. 3.** Knockdown of SHIP1 in vivo using siRNA in the context of miR-155 processing. (A) Schematic of the retroviral vector (MGP-155f) used to deliver siRNA against SHIP1 in miR-155 format. (B) Knockdown of SHIP1 was assayed in RAW 264.7 cells infected with MGP-siSHIP1 or control vector by Western blotting. α-Tubulin was assayed as a loading control. (C) Knockdown of SHIP1 in vivo by retroviral expression of miR-155 (MGP-155, n = 4 mice) or siSHIP1 (MGP-siSHIP1, n = 3 mice) in the hematopoietic compartment was assayed by qPCR using RNA isolated from total bone marrow following 2 months of hematopoietic reconstitution. Relative expression values have been normalized to L32 mRNA. A *P*-value of 0.05 or less using a Student’s *t*-test was considered statistically significant and indicated with an asterisk.

**Knockdown of SHIP1 in the Hematopoietic Compartment Causes a MPD Similar to that Observed in Mice Expressing miR-155.** Mice expressing miR-155, siSHIP1, or control vectors were next studied to determine their impact on hematopoietic populations after 2 months of reconstitution. Both miR-155 (human and mouse sequences) and siSHIP1 caused similar MPD phenotypes in the bone marrow and spleen compared to control vectors (Figs. 4 and 5). Gross examination revealed a miR-155- or siSHIP1-dependent splenomegaly and pale coloring of the bone marrow (see Fig. 5A and unpublished observations). Flow cytometry detected an increase in CD11b+ (Mac1+) myeloid populations in the bone marrow and spleen (see Figs. 4A and 5A). The percentage of Ter119+ erythroid precursor cells was increased in the spleen and decreased in the bone marrow, while the percentage of B220+ B cells was decreased in both the spleen and the bone marrow (see Figs. 4A and 5A).

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**cells at this time point (see Fig. 2C).** SHIP1 mRNA levels reflected similar differences between WT and miR-155-/- BMMs following 24 h of LPS treatment (Fig. 2D). These results are consistent with miR-155 repressing SHIP1 expression after their induced coexpression by LPS, and demonstrate that this can occur under physiologically relevant conditions in primary cells.

**Knockdown of SHIP1 in Vivo Using siRNA in the Context of miR-155 Processing.** Having identified SHIP1 as a direct target of miR-155, we next determined if specific knockdown of SHIP1 levels could recapitulate the miR-155 MPD phenotype in mice that we recently described (7). Although this phenotype is predicted by the MPD observed in SHIP1-/- mice (26–28), we wanted to confirm the presence of pathological myeloproliferative conditions in miR-155- and siSHIP1-expressing cells, which are GFP positive, are responsible for the increased myeloid populations (CD11b+) in the bone marrow (see Fig. 4C).

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these expanded myeloid populations in both cases. Thus, miR-155 expression and specific SHIP1 knockdown in the hematopoietic system triggers marked extramedullary hematopoiesis, a likely consequence of the dysregulated blood cell development in the bone marrow.

**Discussion**

Similar to miR-155, many proteins have evolved to regulate immune cell function and cause disease upon their dysregulated expression. Among such proteins, the inositol phosphatase SHIP1 is expressed in the hematopoietic system and has a broad impact on the biology of different hematopoietic cell types (30). SHIP1 functions at the molecular level by hydrolyzing the 5’ phosphate of Phosphatidylinositol (P1)-3, 4, 5-P3 to generate P1-3,4-P2, a process that blocks PI3K-mediated membrane localization of certain PH domain-containing signaling molecules, such as Akt and PLCγ (31–33). Consequently, mice with a global SHIP1 deficiency develop a MPD characterized by increased GM populations, and decreased B-lymphocyte numbers. This condition is thought to occur as a result of its role as a negative regulator of signaling by growth factors and other immune receptors (30, 34). Moreover, knockout of SHIP1 in B lymphocytes causes spontaneous germinal center formation and antibody class-switching (30, 35, 36), while a SHIP deficiency in T cells skews peripheral T lymphocytes toward Th1 and away from Th2 in response to an immune challenge (37). Thus, SHIP1 impacts the same cell types that express miR-155, and plays an opposing role in many cases. In the present study, we identify and characterize a direct link between miR-155 and SHIP1, whereby miR-155 can directly repress expression of SHIP1 and thereby impede its function.

The connection between miR-155 and SHIP1 has implications for normal immune physiology, as described above, as well as pathological conditions, such as cancer. Our present findings demonstrate a strong correlation between the MPDs caused by miR-155 expression or specific knockdown of SHIP1. Both perturbed the hematopoietic process, resulting in increased GM cell populations, reduced lymphocyte numbers, impaired erythropoiesis, and extramedullary hematopoiesis in the spleen. Therefore, miR-155 repression of SHIP1 may prove to be a contributing factor to human MPDs and myeloid leukemias, where miR-155 has been shown to be over-expressed (7, 23). Of note, SHIP1 is mutated in some acute myeloid leukemia patients, where loss of function has been implicated in the oncogenic process (38, 39). As miR-155 levels are elevated in certain B-cell lymphomas (13–17), and because SHIP1 is a negative regulator of B-cell activation and survival (30, 35, 36), the miR-155-SHIP1 axis should also prove to be of relevance to B-cell malignancies. Two recent abstracts suggest that this is the case (40, 41). It is also plausible that virally encoded orthologs of miR-155 (42–44), or miR-155 induction by viruses such as Epstein-Barr virus (45), can decrease SHIP1 expression en route to B-cell activation and
transformation. Interestingly, the seed region of these orthologs is identical to that of miR-155, while the flanking regions have diverged dramatically from the mature mammalian miR-155 sequence. This would indicate that certain viral miRNAs have specifically evolved to repress seed-dependent targets of miR-155, such as SHIP1.

An important question in the field is whether miRNAs function through repression of a single or a few targets, or via the cumulative impact of repressing large sets of targets. To date, several putative targets of miR-155 have been predicted through bioinformatic and proteomic approaches (46), indicating that there may be great complexity underlying miR-155 function. It may be that the MPD caused by miR-155 also involves other targets than SHIP1, but our studies show that knockdown of SHIP1 alone can phenocopy many of the effects of miR-155. MiR-155 has various roles in different cell types and physiological situations and analysis of particular specific targets, such as PU.1 (22), AID (47, 48), SOCS1 (49), and now SHIP1, suggest that individual targets likely make significant contributions to miR-155 function in a context-dependent manner. For example, PU.1 is repressed by miR-155 and its over-expression has been shown to recapitulate the Ig class-switching defect observed in miR-155−/− B cells (22). AID is repressed by miR-155 in B lymphocytes, which has been elegantly demonstrated via germline mutation of the miR-155 seed region using TargetScan (24). The AID studies also provide evidence that miR-155 targeting of AID impacts Ig class-switching and the rate of c-myc translocations in B lymphocytes. SOCS1 targeting by miR-155 has just recently been shown to impact T-regulatory cell homeostasis (49). Similar observations have been made for specific targets of miR-150 (11, 50), miR-223 (6), and miR-17–92 (10, 12), suggesting a common theme of a few significant targets making dominant contributions to miRNA function. Ultimately, germ-line mutation of miRNA target-seed sequences within the 3′ UTR regions of specific targets, as has been done for AID (47, 48), will provide the strongest argument for relevance.

As both miR-155 and SHIP1 regulate critical and overlapping functions of a variety of cell types of the immune system, therapeutic manipulation of this molecular interaction may prove to be useful in the treatment of diverse pathological conditions, including infection, cancer, and autoimmunity.

Materials and Methods

Cell Culture. Raw 264.7 macrophage and 293T cells were cultured in complete DMEM with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin. For generation of BMMs, bone marrow cells were isolated from the tibias and femurs of mice as previously described (19). All cells were cultured in a humidified incubator with 5% CO2 at 37°C. Primary macrophages were stimulated using fresh DMEM containing 10 ng/ml LPS at 10 μg/ml for 7 to 10 days.

Sequence Alignments. SHIP1 3′ UTR sequences from human, mouse, rat and dog were obtained and aligned with each other and with the miR-155 seed region using TargetScan (24).

DNA Constructs. Retroviral constructs MG and MG-155 (human sequence) were described previously (19). Oligonucleotide sequences used to generate new constructs are provided as supplemental data [supporting information (SI) Table S1]. The MGP-155 expression cassette containing the mouse miR-155 hairpin sequence and flanking regions was cloned from cDNA made from mature human miR-155, while the flanking regions have diverged dramatically from the mature mammalian miR-155 sequence. This would indicate that certain viral miRNAs have specifically evolved to repress seed-dependent targets of miR-155, such as SHIP1.

For reporter assays, the Picalm 3′ UTR was cloned as described previously (7). The mouse SHIP1 3′ UTR was amplified by PCR from cDNA derived from mouse RAW 264.7 cells. This PCR product was cloned into pmiReport (Ambion) using SpeI and HindIII. Assembly PCR was used to mutate the 6-nucleotide miR-155 seed region. A 2-mer control insert and the IRAK1 3′ UTR (used as negative control) were described previously (7).

Luciferase-Beta Gal Reporter Assays. Experiments were performed as previously described using FUFU, FUW-155, β-gal expression vector, and pmiReport vectors transfected into 293T cells (7). Transfections were carried out with TransIT 293 (Mirus). Data were normalized for transfection efficiency using a β-gal reporter and is represented as the ratio of luciferase activity of the transfection containing FUW-155 to that of the transfection containing FUW.

RNA Quantification. SYBR Green-based quantitative real-time PCR was conducted using the 7300 Real-time PCR system (Applied Biosystems) to assay BIC, SHIP1, and L32 mRNAs with Primers designed to the human miR-155 seed mutant (see Table S1).

Flow Cytometry. Fluorescein-conjugated monoclonal antibodies specific to B220 (B220B6) were used to stain B-cells and RBC-containing bone marrow cells that were washed and fixed with paraformaldehyde (1% final). Stained cells were assayed using a BD FACSCalibur flow cytometer (BD) and further analyzed with FlowJo software.

Morphological Assessment of Hematolymphoid Tissues. Histological and cytological samples were prepared and analyzed as described previously (7).

Statistical Tests. All statistical tests were performed using Microsoft Excel.

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