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Serc homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein β are targeted by miR-155 in B cells of Eμ-MiR-155 transgenic mice

We showed that Eμ-MiR-155 transgenic mice develop acute lymphoblastic leukemia/high-grade lymphoma. Most of these leukemias start at approximately 9 months irrespective of the mouse strain. They are preceded by a polyclonal pre-B-cell proliferation, have variable clinical presentation, are transplantable, and develop oligo/monoclonal expansion. In this study, we show that in these transgenic mice the B-cell precursors have the highest MiR-155 transgene expression and are at the origin of the leukemias. We determine that Src homology 2 domain-containing inositol-5-phosphatase (SHIP) and CCAAT enhancer-binding protein β (C/EBPβ), 2 important regulators of the interleukin-6 signaling pathway, are direct targets of MiR-155 and become gradually more downregulated in the leukemic than in the preleukemic mice. We hypothesize that miR-155, by down-modulating Ship and C/EBPβ, initiates a chain of events that leads to the accumulation of large pre-B cells and acute lymphoblastic leukemia/high-grade lymphoma. (Blood. 2009;114:1374-1382)

Introduction

B-cell lymphomas represent the majority of the lymphoid neoplasms, and despite important steps made toward the understanding of their molecular basis, little is known about the initiating factors. Recently, a newly discovered class of noncoding RNAs called microRNAs, with roles in the posttranscriptional regulation of mRNAs, has emerged as possibly implicated in cancer pathogenesis.1

We and other groups identified alterations of miRNA expression in various cancers ranging from leukemias2 to solid tumors.3 Whole genome expression assays revealed that microRNA deregulations are tumor specific, and that they could predict the clinical course and outcome. Therefore, it seems that microRNA profiling could constitute a “signature” useful to differentiate various types of tumors and predict their possible clinical course.2 Because one miRNA can control the translation of multiple mRNAs simultaneously, a single modification of their expression could reverberate on various biologic levels.

Among the many miRNAs identified as being involved in cancer, some seem to be more relevant. The list includes MiR15a/16-1 down-regulated in chronic lymphocytic leukemia,4 MiR21 overexpressed in breast cancer and a plethora of other solid malignancies,3,5 and the cluster MiR17-92, which seems to act as an oncogene in hematologic malignancies and possibly also in solid malignancies.6 In addition, MiR-155 was found overexpressed in several types of B-cell leukemias/lymphomas,2,7,8 with the highest level of expression in diffuse large B-cell lymphomas.7

Despite the large number of in vitro studies designed to understand microRNAs’ roles in cancer, there are only very few animal models available that could help us assess the impact of their deregulation in complex organisms.

We have previously reported9 the creation of the Eμ/VH MiR-155 transgenic mice. They present a block at the pre-B stage of B-cell differentiation at an early age (3-4 weeks), followed by a pre-B-cell proliferation that later translates into frank acute lymphoblastic leukemia/high grade lymphoma.

Now we present a detailed characterization of their immunophenotype and describe a possible mechanism for these leukemias. Initially, all transgenic mice show an increase of the B220+ immunoglobulin (Ig)M+ population; later on, these cells tend to lose some of the B220 surface antigen expression and become B220−; thus, the leukemias studied exhibit a mixed immunophenotype characterized by B220−IgM− and B220−IgM+ (most likely due to some degree of differentiation of some of the malignant clones of the pre-B cells of origin). Moreover, all transgenic mice exhibit an increase of the myeloid line and a reduced T-cell population with no significant changes in the CD4/CD8 ratio.

MiR-155 transgene was found differentially expressed in various hematopoietic organs and B-cell subpopulations, reaching a maximum in the bone marrow and pre-B cells.

To understand the role of MiR-155 in the B-cell differentiation and leukemogenesis, we focused on 2 proteins known to be both key molecules in B-cell maturation/activation and predicted targets of this miR.

Src homology 2 domain-containing inositol-5-phosphatase (SHIP) is a negative regulator of the cell signaling in the immune system.10 This phosphatase is also thought to be implicated in the
B-cell maturation because it has a differential expression in the pro-B compared with the pre-B stage.\textsuperscript{11,12} Irradiated mouse bone marrow reconstituted with \textit{Ship}\textsuperscript{−/−} hematopoietic cells shows a reduction in the immature and mature forms of B cells.\textsuperscript{11,12} Moreover, \textit{Ship}\textsuperscript{−/−} cells were more viable and had better survival due to the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways.\textsuperscript{11}

CCAAT enhancer-binding protein \(\beta\) (C/EBP\(\beta\)) nuclear factor–interleukin (IL)-6 is another mediator of the IL-6 signaling pathway; IL-6 activates C/EBP\(\beta\) through the phosphorylation of MAPK.\textsuperscript{13,14} In addition, C/EBP\(\beta\) expression varies during differentiation of myeloid and plasma cells,\textsuperscript{15-17} suggesting its involvement in myeloid and lymphoid maturation. Mice lacking C/EBP\(\beta\) showed a lymphoproliferative disorder nearly identical to a rare and aggressive form of human Castelman disease, characterized by lymphoid follicular hyperplasia due to an acceleration of the B-cell maturation and accumulation of IgG1-secreting B lymphocytes.\textsuperscript{18} A knockout mouse model for \textit{miR-155} recently published\textsuperscript{19} showed a depletion of B lymphocytes in the germinal center, whereas a knockin mouse for the same gene, in which the endogenous copies are constitutively expressed under the control of a CD21 promoter (active in mature B cells only), presented an accumulation of IgG1-secreting B lymphocytes.

In this study, we show that SHIP and C/EBP\(\beta\) protein levels are diminished in the \textit{MiR-155} transgenic pre-B lymphocytes. SHIP is gradually down-regulated in the preleukemic and leukemic pre-B cells, whereas C/EBP\(\beta\), even though it maintains the same level of expression in the preleukemic mice, is markedly diminished in the leukemic pre-B cells. Thus, we conclude that \textit{MiR-155} transgene has a maximum of expression in the pre-B stage, in which it down-regulates both \textit{Ship} and C/EBP\(\beta\), 2 inhibitors of IL-6. This blocks the B-cell differentiation and might also induce a reactive proliferation of the myeloid line. The B-cell precursors, arrested in their development, proliferate and are at the origin of malignant leukemias with a mixed immunophenotype B220\(^{hi}\)/IgM\(^{−}\) and B220\(^{lo}\)/IgM\(^{+}\).

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### Methods

#### Transgenic mice

\textit{MiR-155} transgenic mice have been generated on both backgrounds, C57BL/6 and FVB, as previously described.\textsuperscript{4} Transgenic mice were housed and killed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Ohio State University and local and federal regulations.

#### Transplants

Fresh splenocytes were collected from transgenic leukemic mice and resuspended in phosphate-buffered saline (PBS). Dilutions of 10\(^6\) cells/mL were prepared and injected intraperitoneally in syngeneic mice (on average, 5 syngeneic mice were injected for each leukemic mouse). An equal number of wild-type mice, strain-, age-, and sex-matched, were then injected with the first group of mice. The transplanted mice got sick and died.

#### Histology, immunohistochemistry, and flow cytometry

All sick mice were killed and weighed, together with littermate controls; spleens, liver, kidneys, lungs, and lymph nodes were collected; spleens were weighed. Fragments of spleens were fixed in 10% buffered formalin, paraffin included, stained with hematoxylin and eosin, and studied with a Olympus BX41 light microscope. Images were acquired with the Olympus software. Unstained slides were prepared and later immunohistochemically stained with in-house manufactured antibodies: CD20, CD79a, CD3, CD43, IgM, and \(\kappa\) and \(\lambda\) light chains (Children’s Hospital). Single-cell suspension of splenocytes was depleted of mature red blood cells by hypotonic lysis (0.165 M NaCl) and stained with the following conjugated antibodies: anti-B220, anti-CD19, anti-CD138, anti-IgM, anti-IgD, anti-CD3, anti-CD4, anti-CD8, anti-CD11b, and anti-IL-4 (all antibodies were from BD Pharmingen). Flow cytometry was carried out on a BD FACScalibur, and data were analyzed using the BD FACS Convert 1.0 for Mac software. For cell sorting, the splenocytes were stained with anti-B220 and anti-IgM; the cell sorting was carried out on a BD Aria. Three populations were selected: B220\(^{+}\) only, B220\(^{+}\)/IgM\(^{+}\), and B220\(^{−}\)/IgM\(^{−}\).

#### Southern blot for VDJ rearrangement

A probe was amplified in the JH4 fragment of the IgH region on the mouse genomic, using the following primers: forward, 5’-TGAAGGATCTGCGA-GAACCAGAA-3’, reverse, 5’-TGCAATGTCAAGAAAATCCCAT-3’, and then cloned into a TOPO TA vector (Invitrogen).

Spleens of the transgenic and wild-type mice were dissociated between frosted slides in PBS, treated with ammonium chloride for the erythrocyte lysis, centrifuged, and resuspended in PBS. DNA was extracted from the splenocytes with phenol-chloroform and digested with EcoRI and PstI. Digested DNA was then separated on a 1% agarose gel, blotted on a HyBond N+ membrane (GE Healthcare), hybridized overnight with the JH4 probe radioactively labeled with 32P (with Klenow enzyme from PrimeIT II Random Primer Labeling kit; Stratagene), and exposed to a phospho-image screen and processed using a Typhoon scanner (GE Healthcare).

#### Western blots

For Western blot on total splenocytes, cells were isolated from sick and wild-type mice, by dissociating the spleens in 2 frosted slides; red blood cells were lysed with ammonium chloride, and proteins were extracted from the remaining white blood cells with a lysis buffer containing Tris-HCl, pH 7.5 (30 mM), NaCl (150 mM), 10% glycerol, and 1% Triton X-100 and protease inhibitors (Roche; 1 dose/mL buffer). A total of 80 μg proteins was then loaded, separated on a 4% to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel in an electrophoretic X-Cell Sure Lock machine, and blotted on polyvinylidene difluoride membranes (Bio-Rad); membranes were incubated overnight with a polyclonal anti–rabbit SHIP-1 primary antibody at a final concentration of 1/100 (Millipore) and polyclonal anti–rabbit C/EBP\(\beta\) 1/200 (Santa Cruz Biotechnology). The following day, membranes were incubated with an anti-rabbit secondary antibody for 1 hour. Signal was detected with an enhanced chemiluminescence kit (Amersham).

For the immunoblot on sorted splenocytes, cells were harvested, washed once with ice-cold PBS, and directly lysed in Laemmli buffer (Bio-Rad; 10 μg cells/50 μL). Western blotting was performed according to previously published protocols.\textsuperscript{20,21} Antibody-reactive proteins were detected with horseradish peroxidase-labeled sheep anti–rabbit, –mouse, and/or –goat Ig sera and enhanced chemiluminescence (Amersham). Proteins were analyzed in 4% to 15% SDS-PAGE (Bio-Rad) using reducing conditions. Monoclonal and polyclonal antibodies used were as follows: polyclonal rabbit sera anti–SHIP-1 from Millipore; polyclonal rabbit anti-C/EBP\(\beta\) antibodies and polyclonal goat anti-actin antibodies from Santa Cruz Biotechnology; and mouse monoclonal anti-GRB2 antibodies from Santa Cruz Biotechnology.

All Western blot films were scanned with a Personal Densitometer SI (Molecular Dynamics) scanner, and protein expression bands were analyzed with Image Quant (Molecular Dynamics) software as follows: scanned bands were selected and analyzed in terms of quantity of the black hue. Results were displayed on graphics. Then the intensity values of the bands for the proteins of interest were divided by the intensity values of the normalizing bands. The ratios obtained this way were then compared.

#### Luciferase activity assay

A fragment of 584 basepairs of the mouse \textit{Cebpb} 3′-untranslated region (UTR) and another one of 1295 basepairs of the mouse \textit{Ship} 3′-UTR, both
containing complementary sequences to mmu-MiR-155, were amplified from mouse genomic DNA and cloned in the luciferase pGL3 control vector (Promega) in the XbaI restriction site. These constructs were then used to synthesize a MiR-155 mutant seed, with the help of the Stratagene QuikChange XL Site-Directed Mutagenesis kit. The mutagenic primers used for Ship were as follows: 5'- GGTGGGTTCTGAAGAGTTTTTA- AAAAAAAATAAGAAAACCATCGG- 3' for the forward and 5'- CCGATGGTTTTCTTTATTGCTTTTTAAACATCTCGAGACC- ACC-3' for the reverse; for C/EBPB the mutating primers were as follows: 5'- GATAATTTGAGAAACCTTTTCGGTTTCAGCAGAAATAGGAAGACA- 3' for the forward and 5'- TGTCTTCATTATTGCTC- GAAACGGAAAAGGTTCAAAA TA TAC-3' for the reverse. We used the same mutation as described by Vigorito et al. All mutations were confirmed by sequencing.

To test whether Ship is a target for miR-155, we cotransfected 293 cells using Lipofectamine 2000 (Invitrogen), as follows: 6 samples were transfected with 0.8 μg Ship 3'UTR cloned in pGL3 control vector and 100 mM mmu-miR-155 precursor (Ambion), and 6 other samples with 0.8 μg Ship 3'UTR cloned in pGL3 control vector and 100 mM miR negative control I (Ambion) instead of miR-155. We also cotransfected 6 samples with 0.8 μg mutant Ship 3'UTR and 100 mM mmu-miR-155, and 6 samples with 0.8 μg mutant Ship 3'UTR and 100 mM miR negative control I instead of miR-155 (Ambion); the same experiment was run for CEBPB. All wells were also transfected with 0.05 μg/well control Renilla luciferase vector pRL-TK (Renilla luciferase, Promega). Cells were lysed after 24 hours and analyzed for luciferase and Renilla luciferase activities using the Dual Luciferase Reporter Assay kit (Promega) on a GLOMAX (Promega) microplate luminometer.

**Results**

Eμ/Vh miR-155 transgenics of both strains (FVB and C57BL/6) develop leukemias/lymphomas transplanted into syngeneic mice

MiR-155 transgenic mice were generated, as previously described. We expanded the colony to 500 transgenics and observed it for more than 2 years.

As reported, all transgenics display enlarged spleens as early as 3 to 4 weeks of age; some of them start showing clinical signs of malignant disease at 4 to 6 months (supplemental Figure 1A-C); however, we noted that more often the onset of the disease was at 9 months; in most cases, the mice were dead before 1 year of age (see survival chart, supplemental Figure 1A).

Malignant lymphocytes from both strains were isolated from leukemic spleens (supplemental Figure 1D) and injected in 40 syngeneic mice that started to show signs of disease (ruffled fur, hunched posture; see supplemental Figure 1C) approximately 4 weeks after the injection (see survival chart for the FVB strain transplants in Figure 1B; the C57BL/6 exhibited a very similar profile; data not shown); the leukemias in the syngeneic mice were clinically, histologically, and immunohistochemically identical to the primary ones. There were some differences between the FVB (400 mice taken into the study) and C57BL/6 transgenic mice (∼100 mice considered for our study) in terms of the phenotype exhibited. The FVB transgenics got sick slightly earlier (by 13 months, only 26% of the group was still alive; see survival chart, Figure 1A) and presented from a very early age an important increase in the size of the spleen, whereas the C57BL/6 initially had a less prominent splenomegaly and presented signs of disease a few months later than the FVB mice (60% of the group was alive at 13 months, and 20% at 18 months; see survival chart, Figure 1A).

**Leukemias in the miR-155 transgenic mice have a mixed B220+IgM−/IgD− and IgM+/IgD+ phenotype**

Histologically, the transgenic spleens exhibited the effacement of their architecture by a diffuse lymphoid proliferation composed mainly of large cells with marked atypia and numerous atypical mitoses. This lymphoproliferation was also present.
extensively in the bone marrow with the complete obliteration of their histology, and in a significant number of cases also in other organs (e.g., mouse no. 764 showed extended liver infiltration, whereas mouse no. 384 had a widespread infiltration in both kidneys and liver). All sick mice exhibited marked lymphadenopathy.

Spleens, livers, and lymph nodes of sick mice immunohistochemically stained showed marked lymphoproliferation positive for pre–B-cell immunomarkers, such as CD20 and CD79a, and negative for T-cell markers, such as CD3 (data not shown). The proliferating lymphocytes also stained positively for intracytoplasmic chains and negative for surface IgM (data not shown). κ and λ chains were also absent.

The flow cytometry showed an increase in the pre–B-cell population of approximately 7.45% plus or minus 0.14% (marked B220+/IgM−) in the preleukemic mice, whereas the leukemic cells exhibited a mixed phenotype B220+/IgM− and B220+/IgM+ (see Figure 2A). The percentages of B and pre-B cells did not change when we marked them for CD19 and IgD instead of B220 and IgM (data not shown). The T-cell population was diminished in all transgenics in various degrees of 5% to 30% (supplemental Figure 2). The T helper cell 1 population stained CD4+ and IL-2+ was unchanged (data not shown). Surprisingly enough, the myeloid line (CD11b+) was also increased in the transgenics (to 16.52% ± 2.4%) compared with the wild-types (Figure 2B).

**Figure 2.** MiR-155 preleukemic transgenic mice are characterized exclusively by an expansion of the pre–B-cell population, whereas the MiR-155–induced leukemias display a mixed B220+/IgM− and B220+/IgM+ immunophenotype. The myeloid lineage was equally expanded in all transgenics, preleukemic and leukemic alike. (A) Flow cytometry for B220 and IgM shows an increase of the B220+/IgM− population in the preleukemic spleens compared with the wild-type ones; the leukemic spleens exhibit a mixed B220+/IgM− and B220+/IgM+ immunophenotype. (B) Flow cytometry for CD11b shows an important increase of the myeloid lineage in the transgenic spleens (preleukemic and leukemic) compared with the wild-types.

TaqMan assay detected the highest levels of miR-155 transgene expression in the bone marrow and the B-cell precursors

TaqMan assay was used to quantify the miR-155 transgene expression in various hematopoietic organs as well as in different flow cytometry-sorted B-cell subpopulations. We identified miR-155 to be more expressed in the bone marrow (~2.5 times more) compared with the spleen, a possible indication of the preferential transcription of this transgene in the precursor rather than the mature lymphoid cells (see Figure 3A). The splenocytes of leukemic, preleukemic, and wild-type mice were sorted by flow cytometry in 2 subpopulations: pre-B (B220+/IgM−) and mature B (B220+/IgM+) cells. TaqMan assay performed on RNA extracted from these 2 cell populations indicated miR-155 transgene to be approximately 10 times more expressed in the precursors (B220+/IgM−) compared with the mature lymphocytes (B220+/IgM+);
is down-regulated in the context of transgenic leukemias (Figure 4A-B), confirming that, indeed, MiR-155 control of line F8, whereas tg2 belongs to the founding line F10. (B) TaqMan assay indicated that Ship is polyclonal, with the emergence of one or few dominant clones. In supplemental Figure 3B (in which the genomic DNA was digested with PvuRI), there is a case of leukemia with a rearranged band of approximately 6 kb (blue asterisk), approximately 8 kb (lane 3, red asterisk) of equal intensity to that of the germline that is of approximately 6 kb (blue arrow in supplemental Figure 3A). Because the intensity of the germline bands is much stronger than that of the rearranged bands (supplemental Figure 3A-B). In supplemental Figure 3A (in which the sick splenocyte genomic DNA was digested with EcoRI), there is a case of leukemia with a rearranged band of approximately 6 kb (lane 3, red asterisk) of equal intensity to that of the germline that is of approximately 6 kb (blue asterisk), suggesting that this lymphoproliferation is monoclonal.

Southern blot on diseased splenocytes for the VDJ rearrangement reveals the oligo/monoclonality of the malignant lymphoid population

Southern blot on diseased splenocytes for the VDJ rearrangement of IgH chains (to assess the clonality) showed oligo- and monoclonal rearranged bands (supplemental Figure 3A-B). In supplemental Figure 3A (in which the sick splenocyte genomic DNA was digested with PvuRI), mouse no. 312 (lane 1) exhibited one extra band of 4 kb, whereas mouse no. 764 (lane 4) had 2 rearranged bands, of 6 kb and 10 kb, respectively (see black arrows, supplemental Figure 3A); 5-kb bands represent the germline heavy chain locus (see red arrow in supplemental Figure 3A); an additional faint band of 3 kb observed in all samples is a cross-hybridization band (see blue arrow in supplemental Figure 3A). Because the intensity of the germline bands is much stronger than that of the rearranged bands, it is quite likely that the disease for the most part is polyclonal, with the emergence of one or few dominant clones. In supplemental Figure 3B (in which the genomic DNA was digested with EcoRI), there is a case of leukemia with a rearranged band of approximately 8 kb (lane 3, red asterisk) of equal intensity to that of the germline that is of approximately 6 kb (blue asterisk), suggesting that this lymphoproliferation is monoclonal.

MiR-155 targets Ship and C/EBPβ, 2 negative regulators of IL-6 lymphoid signaling pathway

SHIP (Inpp5D) is a phosphatase differentially expressed in B-cell precursors\(^{11}\). Ship\(^{-/-}\) knockout mice display a block in B-cell differentiation at the pre-BI stage, whereas Ship\(^{-/-}\) splenocytes have an enhanced proliferative responsiveness to cytokine stimulation. They also exhibit prolonged survival and increased viability, supposedly due to the activation of PI3K/AKT and MAPK pathways.\(^{11}\) Ship is a predicted target for MiR-155 (TargetScan; see “TargetScan”) and has at least one site of perfect complementarity with the seed of this microRNA in its 3’-UTR sequence (supplemental Figure 4).

We hypothesized that Ship expression could be under the control of MiR-155 and thus down-regulated in the transgenic B-cell precursors. Immunohistochemical staining of leukemic spleens indicated a sharp reduction of Ship expression in MiR-155 transgenic leukemias (Figure 4A-B), confirming that, indeed, Ship is down-regulated in the context of MiR-155 overexpression.

To confirm that Ship is a direct target of MiR-155, a fragment of the Ship 3’-UTR containing the binding seed sequence for MiR-155 was cloned into the luciferase reporter plasmid pGL3, and co transfected in 293 cells, together with a miR-155 mimic. The relative luciferase activity decreased by 42% in the presence of the miR-155 mimic compared with the control (see Figure 4C). The repressive effect of miR-155 was abolished in the presence of the mutated 3’-UTR, demonstrating the direct interaction between the microRNA and the binding site. Thus, Ship is a direct target of miR-155 and, therefore, we expect its expression to be reduced in the Eq- miR-155 B lymphocytes.

C/EBPβ acts as a negative regulator of the IL-6–related signaling pathway in B cells; on the other hand, IL-6 phosphorylates MAPK that activate C/EBPβ.\(^{13}\) C/ebpβ is a predicted direct target of MiR-155 (TargetScan; see supplemental Figure 5).

To verify whether this is biologically true, luciferase activity assays were performed on 293 cells cotransfected with C/ebpβ 3’-UTR, cloned in the luciferase reporter pG3 vector, and a miR-155 mimic; we recorded a decrease of approximately 53% of the relative luciferase activity in the presence of the miR-155 mimic compared with the control (Figure 4D); after mutating 3 bases in the 3’-UTR seed sequence, this decrease was only of approximately 14%, indicating that the binding site is operational. Therefore, C/ebpβ is a direct target of miR-155, and we expect it to be diminished in the MiR-155 transgenic B cells.

SHIP and C/EBPβ present lower expression in the leukemic lymphocytes

Western blot analysis on protein extracts from leukemic and wild-type total splenocytes (Figure 5A) indicated that SHIP was absent in the transgenic samples, whereas C/EBPβ was not expressed in 2 transgenics and had diminished levels in 2 others (Figure 5B). Interestingly, 2 T-cell leukemias arisen in the MiR-155 transgenics had normal levels of CEPB/β (Figure 5B; the T-cell leukemia samples are marked with “T”). To study the correlation between SHIP and C/EBPβ expression level and the degree of malignant lymphoid transformation, we sorted 3 spleen subpopulations by flow cytometry, as follows: immature B cells (B220\(^{-},\) IgM\(^{+}\)), mature B cells (B220\(^{-},\) IgM\(^{-}\)), and non-B cells (B220\(^{-},\) IgM\(^{-}\)). We then assessed SHIP and C/EBPβ expression by Western blot in all these 3 subpopulations in the preleukemic, leukemic, and wild-type mice (Figure 6A-D). Densitometry on the SHIP expression bands in the pre-B cells indicated that this protein is the least expressed in
the leukemic mice, less expressed in the preleukemic, and the most expressed in the wild-type mice (Figure 6A-B). The mature B cells displayed the same gradual reduction of the SHIP expression, ranging from the best expressed in the wild-type to the least expressed in the leukemic. C/EBPβ, on the other hand, did not seem to be less expressed in the preleukemic compared with the wild-type pre-B cells. However, the leukemic pre-B cells exhibited decreased levels of C/EBPβ compared with both the wild-type and preleukemic, as shown by the densitometric analysis of the protein bands on the immunoblot (Figure 6C-D). Unlike SHIP, C/EBPβ seems to maintain the same low level of expression in all mature B cells, without any significant difference between the wild-type, preleukemic, and leukemic mice (Figure 6C). Western blot on the same subpopulations of B cells failed to identify any expression of IL-6, indicating that, most likely, the IL-6 transcription is influenced by SHIP and C/EBPβ down-regulation (data not shown).

Discussion

The Eμ-MiR-155 mice were followed up for approximately 2 years. All transgenic mice developed polyclonal lymphoproliferation, followed by malignant leukemias and lymphomas. All leukemias were transplantable into syngeneic mice with a latency of 1 to 1.5 months. There were only minor differences between the 2 strains (FVB and C57BL/6) in the sense in which C57BL/6 exhibited a slightly longer latency and a milder onset of the disease. Immunohistochemistry showed the malignant lymphoproliferation to be composed of B-cell precursors B220+/CD79a− and IgM−.
irrespective of the strain. Flow cytometry unveiled a more nuanced picture: we, thus, could see that initially the lymphoproliferation was with B220<sup>−</sup> cells, whereas the mature B-cell population did not seem to be affected; in the leukemic mice, however, most of the B cells had diminished expression of the surface antigen B220 (becoming B220<sup>−</sup>), and many of them did not display IgM or IgD. The B220<sup>+</sup>/IgM<sup>−</sup>-positive population was drastically diminished in the leukemic spleens compared with the preleukemic ones. All transgenics showed an unexpected and consistent increase of the myeloid lineage. T-cell population was variably decreased in all transgenics compared with the wild-type mice. TaqMan showed MiR-155 transgene to be expressed at higher levels in the bone marrow compared with the spleen. The pre-B cells exhibited the highest levels of MiR-155 expression. VDJ rearrangement analyses of malignant splenocytes revealed the existence of rearranged bands consistent with the emergence of dominant clones. Noteworthy, Ship<sup>−/−</sup> knockout mice present a block in the B-cell differentiation at the stage of pre-BI (lymphoblast), similar to the MiR-155 transgenic mice, together with a decrease of the bone marrow mature B lymphocytes. Ship is known to be differentially expressed in the pre- and pre-B stages of B-cell maturation. Recently, Nakamura et al demonstrated that the B-cell-defective development in Ship<sup>−/−</sup> mice is due to exogenous IL-6 signaling pathway activation. Experiments with IL-6 recombiant in stromal-free cultures of wild-type bone marrow cells show that this proinflammatory cytokine acts on hematopoietic cells and promotes myelopoiesis while suppressing B lymphoid development. This suggests that SHIP has an important role in the early stages of B lymphoid development, and its action might be due in part to the suppression of the IL-6-initiated cell signaling. Interestingly, Helgason et al demonstrated that Ship<sup>−/−</sup> B cells have an increased phosphorylation and activation of MAPK and AKT, and consequently, are resilient to apoptosis and show a prolonged survival. Moreover, splenocytes from double-knockout mice for PTEN<sup>−/−</sup> and Ship<sup>−/−</sup> have even lower apoptotic rates and longer survival than Ship<sup>−/−</sup> single mutants, demonstrating that, indeed, SHIP is an inhibitor of the AKT pathway. The luciferase assays performed by us indicated that Ship is down-regulated by miR-155. Recently, other groups also have identified Ship as a target of miR-155. O’Connell et al showed that repression of Ship by miR-155 might be at the origin of the myeloproliferative disorder phenotype expressed by hematopoietic MiR-155-overexpressing mice. Moreover, Pedersen et al determined that MiR-155–SHIP interaction is part of the tumor necrosis factor–dependent B-cell lymphoma growth pathway. Immunohistochemical staining showed the absence of Ship expression in the MiR-155–related murine leukemias. Western blot on total splenocytes found SHIP to be drastically decreased in transgenics compared with wild-types. Western blot on flow cytometry-sorted B cells showed SHIP to be down-regulated mainly in the pre-B and mature B cells. The クモアカクサ/Ki-67 expression in different pre-B cells (wild-type, preleukemic, and leukemic) calculated on the previous immunoblot, confirms that C/ebpβ has higher expression in the leukemic pre-B cells than in the preleukemic and wild-type ones.
form of human Castleman disease characterized by polyclonal hypergammaglobulinemia and plasma cell hyperplasia.\textsuperscript{19,21} The inactivation of the IL-6 gene in T cells reverses the abnormal B and plasma cell expansion of the \textit{Cebp}\textsuperscript{−/−} mice, supposedly by lack of induction of the IL-6-dependent B-cell signaling pathway.\textsuperscript{22}

Similar to the \textit{Cebp}\textsuperscript{−/−} mice and IL-6 transgenic, the \textit{CD21cre-MiR-155} knockout mice presented an augmentation of the IgG1-synthesizing cells in spleen and lymph nodes.\textsuperscript{6} The phenotype of both \textit{MiR-155} knockout and knockin mice seems to suggest that \textit{MiR-155} could cause an accelerated B-cell maturation and increased IgG1 cell accumulation at later stages of B-cell differentiation; this could occur in humans as well, because up-regulation of \textit{MiR-155} is commonly observed in diffuse large B-cell lymphoma, especially in the ABC subtype (with activated mature B cells);\textsuperscript{23} Vigorito et al recently suggested that one possible mechanism for the \textit{MiR-155} role in the B lymphocyte maturation might be down-regulation of PU.1.\textsuperscript{22} We believe that \textit{MiR-155} regulates the IL-6-mediated signaling pathway (implicated in B-cell differentiation) by targeting \textit{C/EBP\textbeta}. The down-regulation of \textit{C/EBP\textbeta} causes an IL-6-dependent accumulation of IgG1-positive cells in the knockin \textit{MiR-155} murine model, similar to the PU.1 down-modulation. A deletion of human chromosome 20q in the region where \textit{C/EBP\textbeta} maps has been reported in several cases of myeloproliferative disorders and myelodysplastic syndromes, suggesting that this gene could be a link between myelo- and lymphoproliferative disorders.\textsuperscript{24} Interestingly enough, the \textit{MiR-155} transgenic mice also exhibit invariably an increase of the myeloid lineage. We were able to demonstrate by luciferase assay experiments that \textit{C/EBP\textbeta} is a direct target of miR-155. Two different groups, O’Connell et al\textsuperscript{25} and Yin et al,\textsuperscript{26} have also recently shown that \textit{C/EBP\textbeta} is directly regulated by mir155. Western blot on total splenocytes indicated a decrease of the \textit{C/EBP\textbeta} expression in 2 of our transgenic samples and its total abolition in 2 more others. The immunoblot on sorted splenocytes from wild-type, preleukemic, our transgenic samples and its total abolition in 2 more others. The immunoblot on sorted splenocytes from wild-type, preleukemic, and leukemic animals indicated that, whereas there was no relevant difference in terms of \textit{C/EBP\textbeta} expression between the various mature B cells, leukemic pre-B cells were exhibiting obviously decreased levels of \textit{C/EBP\textbeta} compared with the wild-type and leukemic B-cell precursors. We concluded that \textit{C/EBP\textbeta} down-regulation, similar to that of SHIP, is one of the early events induced by the \textit{MiR-155} overexpression occurring at the pre-BI stage. We believe that in the absence of \textit{C/EBP\textbeta}, cells proliferate faster in preparation for a differentiation that does not occur anymore due to the \textit{Ship} down-regulation.

In conclusion, we show that \textit{MiR-155} transgene expression reaches its peak at the pre-B-cell stage. High levels of \textit{MiR-155} down-regulate \textit{Ship} and free the IL-6 pathway of its inhibitory control, generating a block in the B-cell differentiation and favoring the accumulation of predominantly apoptosis-resistant pre-BI cells. This initiates the leukemogenesis, malignant Ship-deficient lymphocytes proliferating preferentially over the ones that still maintain some \textit{Ship} expression, as shown by the gradual decrease in intensity of the \textit{SHIP} synthesis in leukemic pre-B cells compared with preleukemic ones. \textit{MiR-155} overexpression also down-modulates \textit{C/EBP\textbeta}, liberating the IL-6-dependent signaling pathway of another inhibitory control. This also contributes to the lymphoproliferation identified in the \textit{MiR-155} transgenic mice (Figure 7).

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Authorship

Contribution: S.C. and S.K.S. designed research, performed research, analyzed data, and wrote the paper; C.M.C. designed research, analyzed data, and wrote the paper; I.M.P. designed research; and E.T., R.T., D.P., D.C., P.N., J.H., L.R.K., and A.S. performed research.

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