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MicroRNA activity in B lymphocytes

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Abstract

Gene expression regulation by miRNAs has been reported to control key aspects of B cell differentiation and function (1-14). In this chapter we describe the methodology used to perform a functional screening of a miRNA library to identify miRNAs relevant for mature B cell function in primary mouse B cells. These procedures include the construction of a miRNA library and the expression of individual miRNA clones in spleen B cells, as well as the description of functional assays used to determine the impact of miRNA expression on several aspects of B cell function, such as proliferation, apoptosis and class switch recombination.

Keywords

MicroRNA; B lymphocyte; retroviral transduction; class switch recombination (CSR)

1. Introduction

MicroRNAs (miRNAs) are ubiquitous small noncoding RNA molecules that regulate post-transcriptional gene expression by modifying the stability and/or the translation efficiency of their target mRNAs. The study of miRNA function in lymphocytes is of broad interest since miRNA-driven control has emerged as a key regulatory element in the development and function of T and B lymphocytes (reviewed in (15)). miRNA expression can be modified in B lymphocytes using different methodological strategies that involve the generation of animal models or *in vitro* assays to study miRNA function.

Animal models of genetic gain- and loss- of miRNA function are used to study the role of a particular miRNA in an *in vivo* context. Different animal models to study the function of miRNA in B lymphocytes have been reported: i) miRNA transgenics that overexpress a miRNA in B cell precursors and/or mature B lymphocytes (6,7,14), ii) bone marrow (BM) chimera mouse models in which the mice are reconstituted with transduced BM precursors overexpressing a particular miRNA (1,12), iii) miRNA knock-outs in which the miRNA is depleted in B lymphocytes (2,5,7,13), iv) global miRNA depletion in B cell precursors by specific deletion of dicer, the enzyme required for miRNA processing (4) or Ago2, a component of RNA-induced silencing complex (3) and v) transgenics or knock-ins harboring a mutation in the binding site of a miRNA target allows to assess the effect of the loss of a specific mRNA-miRNA interaction (9,10). In spite of the usefulness of *in vivo* gain- and loss-of-function approaches, *in vitro* experiments can provide in some cases a more convenient and straightforward means to study miRNA function: i) they allow the functional screening of a large number of miRNAs, such as miRNA collections cloned in expression libraries; this type of screening can in turn be used to identify candidate miRNAs for subsequent study through *in vivo* approaches ii) they can be used to identify the specific

mRNA sequence that is targeted by a miRNA, iii) they are considerably more time-saving than the generation of mouse models. Gain of miRNA function in primary B cells and B cell precursors can be accomplished efficiently with retroviral transduction techniques (1,11). Gain of function experiments in B cell lines can be done by retroviral and lentiviral transduction (16,17) as well as by transfection with miRNA duplexes (18,19). B cell lines have also been used for loss of miRNA function assays by transfection with anti-miRNA oligonucleotides (16,18,20).

In this chapter we detail the methodology used to perform a functional screening of a miRNA library in primary B cells. When undertaking this kind of screening, the single most important factor to consider is the availability of *in vitro* assays that will allow testing the functional aspects of interest in a simple, fast and quantitative manner. Here we describe the procedures to clone a miRNA library and express the individual miRNA clones in spleen B cells as well as the readouts used to determine the impact of miRNA expression on several aspects of B cell function, including proliferation, apoptosis and class switch recombination (see an outline in Figure 1).

2. Materials

2.1. miRNA retroviral Constructs

1. Proteinase K buffer: 50 mM Tris pH 8, 200mM NaCl, 10mM EDTA, 1% SDS, 400 µg/ml proteinase K (Roche).
2. Phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma).
3. Absolute ethanol.
4. Specific pre-miRNA primers.
5. PfuUltra high-fidelity polymerase (Roche).
6. DNTPs (Roche).
7. EcoR I (New England Biolabs).
8. Xho I (New England Biolabs).

2.2. Isolation and culture of primary mouse B lymphocytes

1. C57/BL/6J mice (Jackson Laboratory).
2. 70 µm nylon cell strainer (BD Falcon).
3. Phosphate buffered saline (PBS).
4. Fetal calf serum (FCS).
5. AcK lysing buffer (BioWhittaker).
6. Mouse CD43 MicroBeads (Miltenyi Biotec).
7. MS Columns (Miltenyi Biotec).
8. MiniMACS separation unit (Miltenyi Biotec).
9. Anti-mouse B220-PE antibody (BD Pharmingen).
10. B cell medium: RPMI 1640 medium (Sigma) with 10% FCS, 50µM β-mercaptoethanol, 10mM HEPES, 10ng/ml mouse IL-4 (Peprotech) and 25 µg/ml LPS (Sigma-Aldrich).
10. Flat bottomed tissue culture multiwell plates.

11. PKH26GL labeling kit (Sigma-Aldrich).

2.3. Retroviral transduction

1. 293T cells (ATCC).
2. Complete DMEM medium: DMEM with 10% FCS .
3. Complete RPMI medium: RPMI with 10% FCS.
4. Trypsin-EDTA.
5. pre-miRNA and PCL-Eco (Imgenex) DNA preparations.
6. 2x HeBS: 280mM NaCl, 10mM KCl, 1.5 mM Na₂HPO₄, 12 mM (d)-glucose, 50 mM HEPES, pH 7.00 adjusted with 0.5 N NaOH in cell-culture grade H₂O.
7. 2.5 M CaCl₂.
8. Polybrene (Sigma-Aldrich).
9. 0.45 µm filters.

2.4. Quantification of transduction and miRNA processing efficiency

1. Propidium iodide (PI).
2. Trizol (Invitrogen).
3. Chloroform.
4. Molecular biology grade water.
5. SuperScript II reverse transcriptase (Invitrogen).
6. Random primers (Roche).
7. DNTPs (Roche).
8. RNaseOUT (Invitrogen).
9. Specific pre-miRNA primers.
10. Specific mouse GAPDH primers: forward-5'-TGAAGCAGGCATCTGAGGG-3' and reverse-5'-CGAAGGTGGAAGAGTGGGAG-3'-.
11. SYBR green PCR master mix (Applied Biosystems).
12. 96 well qPCR plates (Applied Biosystems).
13. Adhesive film for PCR plates (Applied Biosystems).
14. Real-time quantitative PCR system.
15. Specific miRNA and small RNA endogenous control primers for reverse transcription and subsequent real time PCR (TaqMan MicroRNA Assays; Applied Biosystems).
16. TaqMan microRNA Reverse Transcription Kit (Applied Biosystems).
17. TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems).

2.5. Effect of miRNA over-expression in mature B cells

1. DAPI.

2. Annexin V-APC (BD Pharmingen).
3. Propidium iodide (PI).
4. Staining buffer: PBS 1x, 1% FCS, 1% BSA and 0,01% NaN₃.
5. Anti-mouse IgG1 biotin antibody (BD Pharmingen).
6. Anti-mouse B220-PE antibody (BD Pharmingen).
7. Streptavidin-APC (BD Pharmingen).
8. 7-AAD.
9. Flow cytometer with UV or violet, blue and red laser lines.
10. ModFit flow cytometry analysis software (Verity Software House).

3. Methods

3.1. Design of Retroviral Constructs for microRNA expression

To ectopically express miRNAs in primary mouse B cells we have used a microRNA library comprising 150 individual microRNAs cloned in a retroviral vector that harbors GFP as a reporter protein. DNA fragments corresponding to 150 pre-miRNAs and their flanking 50-bp-long genomic sequences were PCR-amplified and cloned in the pre-miRNA GFP plasmid (11). Using this experimental system, transduced B cells express constitutively the pre-miRNA precursor and GFP under the control of CMV and SV40 promoters, respectively.

When designing vectors for microRNA overexpression several issues should be considered. An important aspect is choosing an appropriate reporter gene. GFP is currently the most frequently used reporter gene due to its suitability for flow cytometry and fluorescent microscopy detection, although other fluorescent proteins such as YFP, Orange and DsRed, can be as well suited for these applications. The use of non fluorescent reporters, such as truncated cell surface molecules, can be considered as an adequate alternative if the assays to be performed in the screening will routinely involve isolation of transduced cells. The second factor that should be considered is the optimization of the pre-miRNA genomic context that is cloned in the vector. We and others (1) have observed that the position and size of genomic context can influence the expression and processing of the pre-miRNA precursor in a miRNA specific fashion. Therefore, when possible it is advisable to design several constructs containing variations of the genomic context included in the retroviral vector and to check pre-miRNA expression in transduced cells (see 3.4) to identify the construct with the highest expression.

To generate miRNA pre-miRNA GFP retroviral constructs:

1. Design PCR primers to amplify the pre-miRNA of interest: obtain pre-miRNA sequences from Sanger miRNA Restry (<http://microrna.sanger.ac.uk/sequences/>), retrieve flanking genomic sequences (<http://www.ncbi.nlm.nih.gov/Genomes/>) and design primers with XhoI/EcoRI sites to amplify the appropriate fragments.
2. Prepare genomic DNA from mouse primary cells by standard procedures.
3. Amplify genomic DNA using XhoI/EcoRI primers and PCR with a high fidelity polymerase.
4. Clone in pre-miRNA GFP XhoI/EcoRI sites. Verify cloning by sequencing.

3.2. Isolation and culture of primary mouse B lymphocytes

3.2.1. Isolation of mouse spleen B lymphocytes by magnetic cell sorting—This protocol describes the isolation of mouse spleen primary B cells using a magnetic depletion technique. Cell magnetic separation is based on the labeling of a cell surface antigen with a specific antibody that is coupled to magnetic beads. A mixture containing labeled and unlabeled cells is passed through a column placed within a strong magnetic field that will retain labeled cells. In the case of magnetic depletion, the cell surface antigen used for labeling is expressed in the cells that are to be removed. In the case of positive magnetic selection, the antigen used for magnetic labeling is exclusively expressed in the cell subset of interest. The former technique has the advantage that the cell subset of interest will not receive any unwanted signaling derived from the binding of an antibody to a cell surface molecule or receptor.

1. Prepare a single cell suspension of mouse splenocytes: isolate the spleen from a C57/BL6 mice (see note 1). Place a 70 μ m nylon cell strainer in a 60×15 mm petri dish with 3 ml of complete RPMI 1640. Place the spleen on the cell strainer and use the plunger of a 2 ml syringe to disgregate the spleen onto the medium. Transfer the cell solution to a 15 ml falcon tube.
2. *Removal of red blood cells from spleen suspension*: centrifuge for 10 min at 400 x g, remove supernatant and resuspend in 1 ml of AcK lysis buffer for 4 min at room temperature. Stop the lysis reaction by adding 10 ml of complete RPMI 1640 medium, spin for 10 min at 400 x g, remove supernatant and resuspend in 2 ml of complete RPMI 1640 medium. Count splenocytes using a hemocytometer (see note 2).
3. *Labeling with CD43 magnetic beads*: resuspend splenocytes in 90 μ l PBS-2% FCS per 10^7 total cells. Add 10 μ l of CD43 MicroBeads per 10^7 total cells, mix well and incubate 15 min at 4 °C with occasional shaking. Wash the cells by adding 10 ml of PBS-2% FCS and spin for 10 min at 400 x g. Remove supernatant and resuspend up to 10^8 cells in 500 μ l of PBS-2% FCS (see note 3).
4. *Magnetic depletion of CD43⁺ cells*: place a MS column in the magnetic field of a miniMACS separator and start the flow of the column by adding 500 μ l of PBS-2% FCS. Apply the CD43-labeled spleen cell suspension to the column, and collect the unlabeled CD43⁻ cells that pass through. Wash the column adding 500 μ l of PBS-2% FCS 3 times and collect the flow in the same tube. Remove the column from the separator and place it in a fresh 15 ml tube. Add 1 ml of PBS-2% FCS and apply the plunger provided with the column to collect CD43⁺ labeled cells (see note 4). Check the purity of the CD43⁻ and CD43⁺ purified fractions by flow cytometry after staining with an anti-mouse B220 labeled antibody. Typically the CD43⁻ fraction contains 95% B220⁺ cells.

¹Spleen mouse isolation should be performed in clean semi-sterile conditions. Spray or submerge the sacrificed mouse in 70% ethanol and extract the spleen in a clean area, and whenever possible, under a laminar flow. After removing the spleen all the procedures should be performed inside a tissue culture laminar flow hood to preserve sterility.

²The number of lymphocytes recovered from a spleen will typically range between $6-8 \times 10^7$ cells. Approximately 50% of splenocytes are B lymphocytes.

³For magnetic selection procedures, keep cells on ice and use pre-cooled solutions to prevent capping of antibodies on the cell surface and non-specific cell labeling.

⁴For selection of B lymphocytes from a single spleen (up to 8×10^7 total cells), use a MS column as indicated in the protocol. For selection of cells from more than one spleen, use one MS column per spleen or a single LS column (with up to 7×10^8 total cells), adapting the volumes as indicated in the manufacturer's instructions.

5. *Culture primary spleen B cells*: proceed to 3.2.2. to label the cells with a cell division tracking dye or culture at 1.3×10^6 cells/ml in B cell medium in flat bottomed multiwell plates (see notes 5 and 6).

3.2.2. Labeling B lymphocytes with cell division tracking dyes—This protocol describes the usage of PKH26 labeling to monitor cell division. PKH26 labeling incorporates aliphatic reporter molecules into the cell membrane lipid bilayer so that subsequent cell division results in sequential halving of fluorescence. PKH26 is a red fluorochrome (maximum emission at 567 nm) that can be excited by a 488 nm laser line, enabling the simultaneous quantification of PKH26 labeling and GFP in conventional flow cytometers. When non- GFP reporter vectors are chosen for miRNA overexpression, labeling with 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) is recommended, due to the higher resolution of this dye in the quantitative analysis of cell division.

1. Wash cells in serum-free RPMI 1640 medium, spin for 10 min at 400 x g and remove the supernatant.
2. Resuspend pellet in Diluent C (1 ml Diluent C per $2 \cdot 10^7$ cells). Add 2 μ l of PKH26/ml of Diluent C.
3. Incubate for 4 min at room temperature.
4. Stop the labeling by adding an equal volume of serum and incubate 1 min at room temperature.
5. Dilute in an equal volume of complete RPMI 1640 medium. Spin for 10 min at 400 x g and remove supernatant.
6. Resuspend in complete medium, transfer to a fresh 15 ml tube and wash by adding 10 ml of complete RPMI 1640 medium, spinning for 10 min at 400 x g and removing the supernatant. Repeat the wash three times.
7. Resuspend in complete RPMI 1640 medium and count cells using a hemocytometer.
8. Plate labeled cells at 1.3×10^6 cells/ml in B cell medium in flat bottomed multiwell plates (see notes 5 and 6).

3.3. Transduction with retroviral supernatants

This protocol describes the procedures for the transient production of retroviral supernatants in 293T cells. Co-transfection of the retroviral plasmid harboring the pre-miRNA together with PCL-Eco plasmid encoding the gag, env and pol proteins required for assembling of retroviral particles enables the production of high-titer supernatants that allow efficient transduction of primary B lymphocytes. Independent transfections for each miRNA retroviral construct and for mock-retroviral construct should be carried-out.

1. *Plate 293T cells (day 1)*: plate 293T cells at 15% to 20% density in complete DMEM medium. Consider the volume of supernatant required for the assay to

⁵B cell viability is dependent on cell to cell interactions, and disturbance of these interactions after the initial set up of the cultures should be avoided. Plan in advance the different analysis time points of B cell cultures, and when possible plate the cells in independent multi-wells so that they will be used for individual analysis. Typically we use a 96-well plate, plated with 2.5×10^5 B lymphocytes, for each analysis time point.

⁶Different stimulation conditions can be used to promote CSR to other isotypes. Stimulation with LPS alone promotes class switch to IgG3, stimulation with LPS, IL-4 and TGF β promotes class switch to IgA, and stimulation with anti-CD40 antibodies and IL-4 can also be used to promote class switch to IgG1. When using alternative CSR stimuli take into account that B cell proliferation, and therefore retroviral transduction, can vary across different stimulation conditions.

choose the adequate multiwell size for 293T plating (see note 7). This protocol describes transfection in 6-well plates, adjust quantities accordingly for other multiwell or plate sizes.

2. *Transfect miRNA and pCL-Eco plasmids in 293T cells by calcium phosphate (day 2)*: Prepare an eppendorf tube (A) with 2 µg of PCL-Eco plasmid, 2 µg of miRNA plasmid and 10 µl of 2.5M CaCl₂. Add milli-Q deionized water to achieve a final volume of 100 µl. Prepare a second eppendorf tube (B) with 100 µl of HeBS 2x. While generating bubbles with a pasteur pipette in eppendorf B, add drop-wise the solution from tube A. Incubate the mixture for 20 min at room temperature to allow for calcium phosphate precipitate formation. Add drop-wise the 200 µl transfection mixture to the 293T plate while swirling the medium.
3. *Remove transfection medium and plate primary B cells (day 3)*: 12 to 16 hours after transfection replace 293T calcium phosphate- containing medium with complete RPMI 1640.
4. Isolate and plate primary B cells in B cell medium as described in 3.2.
5. *Transduce primary B cells (day 4)*: 24 hours after plating primary B cells collect the media from 293T transfected cells and pass it through a 0.45 µm filter. Add 50µM β-mercaptoethanol, 10mM HEPES, 10ng/ml IL-4 , 25 µg/ml LPS and 8 µg/ml polybrene to the filtered retroviral supernatant media. Remove the maximum volume (approximately 80% of total volume) of media from the B cell cultures multiwell plates without disturbing the cells and replace it with the retroviral supernatant media. Save the LPS IL4 containing B cell culture media at 4°C. Centrifuge the B cell culture multiwell plates at 1500 x g at room temperature for 2,5 hours. Culture the cells with the retroviral supernatant.
5. *Remove retroviral supernatant medium (day 5)*: 16 to 20 hours after transduction remove the maximum volume (approximately 80% of total volume) of retroviral medium without disturbing the cells and replace it with 37 °C pre-heated LPS- and IL4-containing B cell culture media that was saved on the previous day.

3.4. Quantification of retroviral transduction and miRNA processing efficiency

The usage of fluorescent proteins such as GFP as reporter proteins of retroviral transduction provides a fast and convenient way to identify transduced cells. However, miRNA processing is a complex multi-step process that involves the sequential cleavage of a long primary transcript (pri-miRNA) into a stem-loop precursor of approximately 70 nucleotides (pre-miRNA), and finally into a 20-22 nucleotide long mature miRNA (reviewed in (21) and (22)). The efficiency of these processes can vary significantly among different pre-miRNAs and cellular contexts, and therefore it is advisable to determine the pre-miRNA and mature miRNA processing efficiency in each specific assay condition. In the following procedures we describe how to determine transduction efficiency by flow cytometry analysis and how to analyse the expression of pre-miRNA and mature miRNA in transduced cells by real-time PCR analysis.

3.4.1. Quantification of transduction efficiency by gene reporter expression—2

days after transduction, remove a sample containing from 5×10^4 to 2×10^5 transduced B cells, spin for 10 min at 400 x g and resuspend in 500 µl of PBS with 2% FCS and 1 µg/ml

⁷Consider the number of B cells to be transduced with each miRNA construct to plate the adequate number of multiwells with 293T cells. Six-, 12- and 24-well plates will yield about 3 ml, 1.5 ml and 0.8 ml of retroviral supernatant respectively. For a screening assay in which a B cell function is assessed through a single end-point time flow cytometry analysis, a single 24-well plate of 293T cells transfected with each miRNA construct will yield enough retroviral supernatant.

propidium iodide. Incubate at room temperature for 5 min and determine the transduction efficiency by flow cytometry analysis of GFP expression in live (propidium iodide negative) cells (see note 8). Typically transduction efficiencies of primary mouse spleen B cells range from 20% to 40%.

3.4.2. Quantification of pre-miRNA processing efficiency in transduced B cells

1. *Design primers for pre-miRNA amplification.* Use *Express Primer* software to design specific real time PCR primers for pre-miRNA amplification. cDNA normalization will be performed by using GAPDH as an endogenous control (GAPDH primer sequences: forward-5'-TGAAGCAGGCATCTGAGGG-3' and reverse-5'-CGAAGGTGGAAGAGTGGGAG-3').
2. *Sort transduced B cells:* 48 to 72 hours after retroviral transduction, collect 5×10^6 to 2×10^7 transduced B lymphocytes, spin 10 min at 400 x g and resuspend in PBS with 2% FCS at 1×10^7 cells/ml. Filter through a 70 μ m filter and sort in a high-speed cell sorter with a 70 μ M nozzle to isolate GFP⁺ transduced B cells. Sort mock-GFP⁺ transduced cells and miRNA-GFP⁺ transduced samples.
3. *Isolate RNA:* Pellet 0.5×10^6 to 3×10^6 sorted GFP⁺ B cells by centrifuging for 10 min at 400 x g. Lyse cells in 1 ml of TRIzol reagent by repetitive pipetting and incubate homogenized samples for 5 min at room temperature. Add 0.2 ml of chloroform. Cap sample tubes securely and shake vigorously for 15 sec. Incubate at room temperature for 3 min and centrifuge at 12.000 x g for 15 min at 4°C. Transfer the upper aqueous phase to a fresh tube and precipitate the RNA by mixing with 0.5 ml of isopropyl alcohol. Incubate for at least 1 hour at -20°C and centrifuge at 12.000 x g for 20 min at 4°C. Remove the supernatant and wash the RNA pellet once with 1 ml of cold 75% ethanol. Centrifuge at 7500 x g for 10 minute at 4°C, remove supernatant and air-dry the RNA pellet for 5 to 10 min. Dissolve RNA in 10 μ l to 30 μ l of RNase-free molecular biology grade water by passing the solution a few times through a pipette tip and incubating for 10 min at 55°C. Measure RNA concentration in spectrophotometer and check RNA integrity in a 0.8% agarose gel. RNAs should be stored at -80°C if not used immediately (see note 9).
4. *Retro-transcribe RNA into complementary DNA (cDNA):* add to a PCR tube 250 ng random primers, 0.5 μ g to 5 μ g of total RNA, 1 μ l of dNTP mix (10 mM each) and ribonuclease-free water to a final volume of 12 μ l. Incubate samples at 65°C for 5 min and then at 2°C for 1 min in a thermocycler. Transfer the tube to ice and add 4 μ l of 5x First-Strand Buffer, 2 μ l of 0.1M DTT and 1 μ l (40 units/ μ l) RNAaseOUT. Mix the contents gently and incubate at 25°C for 2 min in a thermocycler. Then add 1 μ l (200 units) of SuperScript II Reverse Transcriptase and incubate at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min in a thermocycler cDNAs should be stored at -20°C if not used immediately (see note 10).

⁸Primary B cell cultures can be kept alive in culture for a maximum of 6 to 7 days. However, the percentage of dead cells in the cultures is significant from day 3 ($\geq 50\%$) and will steadily increase at later time points. It is therefore important to include a cell viability dye that enables live/dead discrimination by flow cytometry analysis. Several dyes with different excitation/emission spectra (DAPI, propidium iodide, TO-PRO or 7-AAD) can be used for this purpose.

⁹When working with RNA keep clean conditions to avoid degradation by ribonucleases. Clean working surfaces and pipettes with 70% ethanol and RNase decontaminant (such as Ambion's RNaseZap), wear always clean gloves, use filter tips and prepare ethanol dilutions with ribonuclease free water. RNAs should be stored at -80°C and kept on ice during all working procedures. Repetitive RNA freezing and thawing cycles should be avoided.

¹⁰Store cDNAs at -20°C. Thaw cDNAs on ice, and keep them on ice while working. Avoid repetitive freezing and thawing cycles.

5. *Analyze pre-miRNA expression by real time RT-PCR:* for each cDNA sample, prepare 2 different dilutions (typically 1/2 and 1/8 of original cDNA stock). Amplify each cDNA dilution with the specific pre-miRNA pair of primers and with the endogenous control (GAPDH) pair of primers. Set all the reactions in duplicates. Prepare a mix for each amplification type containing 10 μ l of SYBR green PCR master mix, 0.4 μ M sense primer, 0.4 μ M reverse primer and molecular grade water to reach a final reaction volume of 18 μ l per reaction. Add 2 μ l of the cDNA dilutions and 18 μ l of the specific amplification mix per well in 96-well PCR plates. Cover the plate with an adherent film and centrifuge it for 15 min at 1500 x g to eliminate any air bubbles. Run the reaction in a real-time quantitative PCR system using standard amplification conditions (50°C 2 min; 95°C 10min; 40 $\times$ (95°C 15 sec; 60°C 1 min). To analyze the data, first normalize the pre-miRNA amplifications by subtracting GAPDH Ct from premiRNA Ct ($dCT = Ct\text{-premiRNA} - Ct\text{-GAPDH}$). Then normalize amplifications of miRNA-transduced samples to mock-transduced samples ($ddCT = dCT\text{-miRNA RV sample} - dCT\text{-mock RV sample}$) and finally calculate fold expression levels in miRNA-transduced samples versus mock-transduced samples ($=\text{power}(2, -ddCT)$).

3.4.3. Quantification of mature miRNA processing efficiency in transduced B cells—

1. Sort transduced GFP⁺ cells as described in 3.4.2.2.
2. Prepare total RNA as described in 3.4.2.3.
3. Retrotranscribe mature miRNA and an endogenous small RNA with specific primers. Use TaqMan microRNA assays from Applied Biosystems following manufacturer's instructions.
4. Analyze by real time RT-PCR mature miRNA expression in mock versus miRNA transduced cells. Use TaqMan microRNA assays from Applied Biosystems following the manufacturer's instructions. Include the same PCR samples as in 3.4.2.5.

3.5. Determination of the functional effect of miRNA over-expression in mature B cells

Mature B cells are programmed to follow a complex maturation and differentiation program upon antigen recognition. This process involves cellular expansion and two molecular mechanisms named somatic hypermutation (SHM) and class switch recombination (CSR) that serve to generate higher affinity antibodies with various functional specificities (reviewed in (23) and (24)). CSR can be induced *in vitro* in cultures of primary B lymphocytes by stimulation with different combinations of factors (see note 6). We describe below the procedures to quantify the effect of miRNA overexpression in B cell proliferation, survival or CSR in *in vitro* cultures of primary B cells. Analysis of other molecular process that occur upon B cell stimulation, such as S μ and S γ 1 germ line transcription, S μ and S γ 1 mutation and AID expression can be analyzed in sorted samples from mock and miRNA-transduced cells following previously reported experimental procedures (11,25-27).

3.5.1. Determination of miRNA impact on B cell proliferation—

1. Remove cells for proliferation analysis at days 2, 3 and 4 after retroviral transduction of PKH26 labeled B cells. Include in the analysis mock-transduced cells and miRNA-transduced samples. Remove from the culture $\sim 1 \times 10^5$ cells, spin them for 10 min at 400 x g and remove the supernatant.

2. Resuspend in 0.3 ml PBS with 2% FCS and 0.1 $\mu\text{g/ml}$ DAPI to label dead cells. Incubate for 5 min at room temperature (see note 8).
3. Analyze PKH26 fluorescence in transduced (GFP^+) live (DAPI^-) cells by flow cytometry. Laser line used for excitation/emission maxima: DAPI (UV or violet laser/blue emission (max 461 nm)), GFP (blue 488 nm laser/green emission (max 509 nm)) and PKH26 (blue 488 nm laser/ orange emission (max 567 nm)). Include the following “single color” control samples to set up flow cytometry compensation parameters: i) nontransduced, DAPI labeled cells, ii) transduced, non-PKH26 labeled samples and iii) non transduced, PKH26 labeled samples,
3. Analyze data with ModFit flow cytometry analysis software.

3.5.2. Determination of miRNA impact on B cell apoptosis—

1. Analyze cell death in *in vitro* cultures of spleen B cells at days 2, 3 and 4 after retroviral transduction. Include in the analysis mock-transduced and miRNA-transduced samples. Spin $\sim 1 \times 10^5$ cells for 10 min at 400 x g and resuspend them in 1 ml of cold PBS. Repeat the washing step once more and then resuspend the cells in 100 μl of 1X AnnexinV binding buffer.
2. Transfer the solution to a 5 ml FACS tube and add 5 μl of AnnexinV-APC. Vortex gently and incubate for 15 min at room temperature in the dark.
3. Add 300 μl of 1 x AnnexinV binding buffer and propidium iodide (PI) to 1 $\mu\text{g/ml}$ final concentration. Incubate 15 min at room temperature in the dark.
4. Analyze by flow cytometry the percentage of early apoptotic ($\text{AnnexinV}^+ \text{PI}^-$) in transduced GFP^+ cells (see note 11). Laser line used for excitation/emission maxima: GFP (blue 488 nm laser/green emission (max 509 nm)), PI (blue 488 nm laser/orange-red emission (max 617 nm)) and APC (red 633nm laser line/red emission (max 660 nm)). Include single stained samples to setup flow cytometry compensation controls.

3.5.3. Determination of miRNA impact on CSR—

1. Analyze IgG1 class switch recombination efficiency in *in vitro* cultures of B cells at days 2, 3 and 4 after retroviral transduction. Include mock-transduced and miRNA-transduced samples in the analysis. Remove from the culture $\sim 1 \times 10^5$ cells, spin them for 10 min at 400 x g and wash with cold PBS. Spin again for 10 min at 400 x g, remove the supernatant and resuspend the cells in 100 μl of staining buffer with a 1/500 dilution of anti-mouse IgG1-biotinylated antibody. Incubate on ice for 20 min.
2. Wash by adding at least 100 μl of staining buffer, spinning for 10 min at 400 x g and removing the supernatant. Resuspend the cells in 100 μl of staining buffer with 1/500 dilution of streptavidin-APC and 1/200 dilution of anti-B220-R-PE antibody. Incubate for 20 min on ice in the dark.
3. Wash by adding at least 100 μl of staining buffer, spinning for 10 min at 400 x g and removing the supernatant. Resuspend the cells in 500 μl of staining buffer and transfer to a FACS tube.
4. Add 5 μl of 7-AAD and incubate for 20 min on ice to label dead cells.

¹¹GFP expression is partially lost as cells die due to cell membrane permeabilization. To accurately quantify the proportion of transduced cells in late apoptosis ($\text{AnnexinV}^+ \text{PI}^+$) either sort transduced GFP^+ cells prior to cell culture or use a modified GFP version, like farnesylated GFP, that is retained in the cell membrane, as reporter gene of retroviral transduction.

5. Analyze by flow cytometry B220-R-PE versus IgG1-APC staining in GFP⁺ transduced live (7-AAD⁻) cells (see note 8). Laser line used for excitation/emission maxima: GFP (blue 488 nm laser/green emission (max 509 nm)), R-PE (blue 488 nm laser/orange emission (max 578 nm)), 7-ADD (blue 488 nm laser/red emission (max 655 nm)) and APC (red 633nm laser line/red emission (max 660 nm)). Include single stained samples to set-up flow cytometry compensation controls. The expected class switch efficiencies to IgG1 in LPS+IL-4 cultures of primary transduced B cells range from range from 20% to 40% at day 4.

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References

1. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004; 303:83–86. [PubMed: 14657504]
2. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, Rajewsky N, Bender TP, Rajewsky K. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell*. 2007; 131:146–159. [PubMed: 17923094]
3. O'Carroll D, Mecklenbrauker I, Das PP, Santana A, Koenig U, Enright AJ, Miska EA, Tarakhovsky A. A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. *Genes Dev*. 2007; 21:1999–2004. [PubMed: 17626790]
4. Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, Kanellopoulou C, Jensen K, Cobb BS, Merkenschlager M, Rajewsky N, Rajewsky K. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell*. 2008; 132:860–874. [PubMed: 18329371]
5. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M, Bradley A. Requirement of bic/microRNA-155 for normal immune function. *Science*. 2007; 316:608–611. [PubMed: 17463290]
6. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, Croce CM. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 2006; 103:7024–7029.
7. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, Murphy A, Frendewey D, Valenzuela D, Kutok JL, Schmidt-Suppran M, Rajewsky N, Yancopoulos G, Rao A, Rajewsky K. Regulation of the germinal center response by microRNA-155. *Science*. 2007; 316:604–608. [PubMed: 17463289]
8. Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, Das PP, Miska EA, Rodriguez A, Bradley A, Smith KG, Rada C, Enright AJ, Toellner KM, MacLennan IC, Turner M. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity*. 2007; 27:847–859. [PubMed: 18055230]
9. Dorsett Y, McBride KM, Jankovic M, Gazumyan A, Thai TH, Robbani DF, Di Virgilio M, San-Martin BR, Heidkamp G, Schwickert TA, Eisenreich T, Rajewsky K, Nussenzweig MC. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. *Immunity*. 2008; 28:630–638. [PubMed: 18455451]
10. Teng G, Hakimpour P, Landgraf P, Rice A, Tuschl T, Casellas R, Papavasiliou FN. MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. *Immunity*. 2008; 28:621–629. [PubMed: 18450484]
11. de Yébenes VG, Belver L, Pisano DG, Gonzalez S, Villasante A, Croce C, He L, Ramiro AR. miR-181b negatively regulates activation-induced cytidine deaminase in B cells. *J. Exp. Med.* 2008; 205:2199–2206. [PubMed: 18762567]

12. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature*. 2005; 435:828–833. [PubMed: 15944707]
13. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA, Jacks T. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008; 132:875–886. [PubMed: 18329372]
14. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, Henderson JM, Kutok JL, Rajewsky K. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nat. Immunol.* 2008; 9:405–414. [PubMed: 18327259]
15. Xiao C, Rajewsky K. MicroRNA control in the immune system: basic principles. *Cell*. 2009; 136:26–36. [PubMed: 19135886]
16. Inomata M, Tagawa H, Guo YM, Kameoka Y, Takahashi N, Sawada K. MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell lymphoma subtypes. *Blood*. 2009; 113:396–402. [PubMed: 18941111]
17. Huppi K, Volfovsky N, Runfola T, Jones TL, Mackiewicz M, Martin SE, Mushinski JF, Stephens R, Caplen NJ. The identification of microRNAs in a genomically unstable region of human chromosome 8q24. *Mol. Cancer Res.* 2008; 6:212–221. [PubMed: 18314482]
18. Nie K, Gomez M, Landgraf P, Garcia JF, Liu Y, Tan LH, Chadburn A, Tuschl T, Knowles DM, Tam W. MicroRNA-mediated down-regulation of PRDM1/Blimp-1 in Hodgkin/Reed-Sternberg cells: a potential pathogenetic lesion in Hodgkin lymphomas. *Am. J. Pathol.* 2008; 173:242–252. [PubMed: 18583325]
19. Kluiver J, van den Berg A, de Jong D, Blokzijl T, Harms G, Bouwman E, Jacobs S, Poppema S, Kroesen BJ. Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene*. 2007; 26:3769–3776. [PubMed: 17173072]
20. Sampath D, Calin GA, Pudevalli VK, Gopisetty G, Taccioli C, Liu CG, Ewald B, Liu C, Keating MJ, Plunkett W. Specific activation of microRNA106b enables the p73 apoptotic response in chronic lymphocytic leukemia by targeting the ubiquitin ligase Itch for degradation. *Blood*. 2009; 113:3744–3753. [PubMed: 19096009]
21. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* 2004; 5:522–531. [PubMed: 15211354]
22. Chang K, Elledge SJ, Hannon GJ. Lessons from Nature: microRNA-based shRNA libraries. *Nat. Methods*. 2006; 3:707–714. [PubMed: 16929316]
23. de Yébenes VG, Ramiro AR. Activation-induced deaminase: light and dark sides. *Trends. Mol. Med.* 2006; 12:432–439. [PubMed: 16861038]
24. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. *Annu. Rev. Immunol.* 2008; 26:261–292. [PubMed: 18370922]
25. Hernandez IV, de Yébenes VG, Dorsett Y, Ramiro AR. Haploinsufficiency of activation-induced deaminase for antibody diversification and chromosome translocations both in vitro and in vivo. *PLoS One*. 2008; 3:e3927. [PubMed: 19079594]
26. Reina-San-Martin B, Difilippantonio S, Hanitsch L, Masilamani RF, Nussenzweig A, Nussenzweig MC. H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation. *J. Exp. Med.* 2003; 197:1767–1778. [PubMed: 12810694]
27. Nambu Y, Sugai M, Gonda H, Lee CG, Katakai T, Agata Y, Yokota Y, Shimizu A. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science*. 2003; 302:2137–2140. [PubMed: 14684824]

Figure 1. Overview of procedures for the functional screening of a miRNA library in B cells

To assess miRNA function in primary B cells, retroviral supernatants from a miRNA library are used to transduce primary mouse spleen B cells. Transduced cells are identified by the expression of a reporter protein such as GFP. Several aspects of B cell function, such as CSR, proliferation and apoptosis can be used as readouts to determine the functional impact of miRNA expression. The figure shows a representative flow cytometry analysis of GFP expression (top right histogram), IgG1 CSR efficiency analysis (bottom left contour plot), PKH26 proliferation analysis (bottom middle histogram and charts) and apoptosis analysis (bottom right contour plot) in which early (AnnexinV⁺ PI⁻) and late (Annexin V⁺PI⁺) apoptosis cells subsets are identified (gates I and II respectively).