The B2 B-cell lineage and antibodies used

The staining procedure applied sub-classifies the B2 B-cell lineage as follows.[1,2] In Fig. 1a the developmental pathway is depicted graphically. Fraction A (FrA) cells in the BM can contain a subpopulation of T-cell progenitors but also contain cells that are starting to commit to B-cell development.[1–4] The latter cells develop into FrB/C cells that first express Pax5, a transcription factor that suppresses development into other immune cell lineages and induces expression of B-cell–specific genes throughout development until the cells develop into plasma cells.[3] FrB/C cells develop into FrC′, which in turn develop into FrD, and the latter differentiate into FrE. These FrE cells leave the BM and migrate to the spleen and upon arrival they are called transitional-1 (T1) B cells. These develop into T2 and/or T3 cells. It has been suggested that T3 represent an immature end-stage of B-cell development that does not develop any further [5] and that T2 cells are a pre-mature stage that contains precursors for both Fo and Mz B cells.[6] For the experiments presented in the current paper the T2 and T3 populations were pooled together (and designated T2/3 cells) as the staining did not allow enough separation. Fo B cells can leave the spleen and recirculate to the BM were they are distinguished as FrF or recFo B cells (in this paper the last denomination is used). Another population of B cells that can be distinguished in the spleen are the B1 B cells which are though to originate from a different developmental pathway.[7] The following reagents were used for B-cell subset staining and sorting (Fig. S1b and c). CD3ε-PE/Cy7 (clone 145-2C11), Ly-6G/Ly-6C-PE/Cy7 (GR1; clone RB6-8C5), TER-119-PE/Cy7 (clone TER-119), CD11b-PE/Cy7 (Mac1; clone M1/70), CD45R/B220-APC (clone RA3-6B2), CD43-FITC (clone S7), Ly-51-biotin (BP1; clone 6C3), CD21/CD35-PE (clone 7G6), IgM-biotin (clone II/41), and Streptavidin-PE/Texas Red were obtained from BD Biosciences (San Jose, CA). CD24-PE (clone 30-F1), CD23-PE/Cy7 (clone B3B4), and CD93-APC (clone AA4.1) were purchased from eBioscience (San Diego, CA) and IgM-PE was obtained from Southern Biotechnology Associates (Birmingham, AL). Flow-sorting was performed on either a customized Becton Dickinson FACS Aria or a customized Becton Dickinson FACS DiVa (San Jose, CA) utilizing RPMI medium containing 30% FBS as the collection media.

RNA extraction

At the time the experiment was performed it was uncertain whether it was possible to obtain enough sequencing data from relatively low amounts of starting material (for instance, in pooled bone marrow of 10 mice only 0.6 million FrA B cells were present yielding only 1 ug of total RNA; Table S1). It was therefore decided to apply all the RNA isolated for each fraction (varying between 1 and 10.5 ug per developmental stage) on the gel for extraction of the 19–24nt small RNA (smRNA) fractions and to proceed with the generation of the libraries.[8]

Identification of known small RNAs

Analysis of the sequence tags was performed using custom and previously existing algorithms. In summary, the sequence tags obtained were filtered for contaminating synthetic oligonucleotides that were involved in the generation of the library using an algorithm provided by Dr. R. Sachidanandam. The remaining reads were sequentially matched with the NCBI BLAST program [9] against 4 small non-coding RNA databases (miRBase (www.mirbase.org), NONCODE (www.noncode.org), GtRNAdb (lowelab.ucsc.edu/GtRNAdb) and RNAdb (research.imb.uq.edu.au/rnadb)) to count known small non-coding RNA (ncRNA) species. In order to obtain the B-cell miRNAome all sequences were matched with NCBI BLAST against

miRBase V14 mature miRNA and miRNA* databases allowing maximally three mismatches (which could be introduced by miRNA editing) and requiring sequence lengths of 19–24 nucleotides. The expression level of each individual miRNA in each stage was either determined as its frequency within the total pool of miRNAs in that stage or as a normalized sequence count. A normalized sequence count for each miRNA per stage was obtained by calculating the deviation of the miRNA's frequency in a certain stage relative to the average frequency of that particular miRNA throughout all 11 stages and to impose this deviation onto the average number of sequence counts for that particular miRNA throughout all stages. To validate our sequence libraries and the normalization strategy the normalized sequence counts were compared (Pearson's correlation) with results from single miRNA TaqMan assays and Low Density Arrays (LDAs) containing hundreds of single miRNA TaqMan assays per plate (Applied Biosystems, Carlsbad, CA) obtained from biological replicate samples.

Validation of the deep sequencing results by Low Density Array analysis.
Low density array analysis was performed using T1, T2/3, Fo and Mz cells obtained independently (biological replicate samples). We determined the miRNA expression levels in all samples by LDA-qPCR analysis and determined the extent to which each miRNA in T2/3, Fo and Mz cells deviated from the level in T1 cells. We then assigned an artificial miRNA level to the T1 cells as the counts determined by deep sequencing. Using the T1-count as the internal standard we calculated artificial counts to the T2/3, Fo and Mz stages which were consecutively compared to the deep sequencing counts for the T2/3, Fo and Mz stages.

Identification of new miRNAs
To identify new miRNA candidates the deep sequencing output was uploaded to the miRanalyzer Web server.[10] It was decided to exclude hairpins that mapped to >2 loci. Additionally the sequence tags in the libraries were annotated to the mouse genome using SHRiMP.[11] An initial list of 405 new miRNA candidates was narrowed down to 65 by manual inspection of the top100 and the hairpins in the top101–405 that could be lifted over to the human genome (UCSC Genome Browser lift-over algorithm).[12,13] Manual inspection criteria required the presence of sequence tags (custom annotation) on the hairpin coordinates (miRanalyzer), the generation of a hairpin structure upon transfer of the hairpin sequence to the Vienna RNA Websuite RNA-fold algorithm [14] and the expected location of the mature miRNA sequence (and where applicable the miRNA* sequence) with respect to the hairpin structure. In case of miRNA/miRNA* pairs, the sequence with the lowest sum of raw counts over all stages was called the miRNA* sequence. The following criteria were used to validate the new miRNAs: 1) finding of the candidates in multiple stages, 2) re-identification of the candidates by deep sequencing of small RNA from B220+ splenocytes, 3) confirmation by NCode™ SYBR® Green miRNA qRT-PCR (Table S6)(Invitrogen, Carlsbad, CA), and 4) conservation of hairpin and sequence in Homo sapiens. The NCode PCR yields products of ~65bps in case of amplification of mature miRNA sequences and ~100bp products in case of amplification of pre-miRNA sequences containing the miRNA on the 5′-end of the molecule. Wherever we found a product of ~100bp length while the target sequence was positioned on the 3′ end of the pre-miRNA molecule, the miRNA was considered to be not validated. PCR products longer than ~100bps could be pri-mRNA.

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