miRNA profiling of B-cell subsets: specific miRNA profile for germinal center B cells with variation between centroblasts and centrocytes

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MicroRNAs (miRNAs) are an important class of small RNAs that regulate gene expression at the post-transcriptional level. It has become evident that miRNAs are involved in hematopoiesis, and that deregulation of miRNAs may give rise to hematopoietic malignancies. The aim of our study was to establish miRNA profiles of naïve, germinal center (GC) and memory B cells, and validate their expression patterns in normal lymphoid tissues. Quantitative (q) RT-PCR profiling revealed that several miRNAs were elevated in GC B cells, including miR-17-5p, miR-106a and miR-181b. One of the most abundant miRNAs in all three B-cell subsets analyzed was miR-150, with a more than 10-fold lower level in GC B cell as compared with the other two subsets. miRNA *in situ* hybridization (*ISH*) in tonsil tissue sections confirmed the findings from the profiling work. Interestingly, gradual decrease of miR-17-5p, miR-106a and miR-181b staining intensity from the dark to the light zone was observed in GC. A strong cytoplasmic staining of miR-150 was observed in a minority of the centroblasts in the dark zone of the GC. Inverse staining pattern of miR-150 against c-Myb and Survivin was observed in tonsil tissue sections, suggesting possible targeting of these genes by miR-150. In line with this, the experimental induction of miR-150 lead to reduced c-Myb, Survivin and Foxp1 expression levels in the Burkitt's lymphoma cell line, DG75. In conclusion, miRNA profiles of naïve, GC and memory B cells were established and validated by miRNA *ISH*. Within the GC cells, a marked difference was observed between the light and the dark zone.

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MicroRNAs (miRNAs) are a class of small RNAs of 19–23 nucleotides that were discovered less than two decades ago.^{1,2} When incorporated into Argonaute proteins to form the RNA-induced silencing complex, miRNAs can negatively regulate genes at the post-transcriptional level by either triggering translational repression or direct cleavage of mRNAs.³ miRNA expression can be highly tissue-specific⁴ and is known to regulate many cellular processes, including proliferation, apoptosis, cell cycle, differentiation and hematopoiesis.^{5–8}

The distinct and dynamic expression pattern of miRNAs during hematopoiesis reflects the importance of this class of small RNAs in determining the various states of differentiation

of hematopoietic cells.^{9–12} Alteration in the expression level of merely one miRNA in hematopoietic stem cells can skew the differentiation process toward a specific hematopoietic cell type.¹³ For this reason, it was assumed that aberrant expression of miRNAs could be involved in the development of hematopoietic malignancies. Indeed, the specific involvement of a number of miRNAs not only in hematopoietic development, but also in lymphomagenesis, has been the subject of a growing number of studies in the past few years.

c-Myb, a transcription factor known to play a key role in B cell maturation, has been shown to be a direct target of miR-150.¹⁴ Ectopic expression of miR-150 in hematopoietic stem

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cells leads to the blockage of the transition of pre-B to pro-B stage.¹⁵ The PU.1 transcription factor, essential in hematopoietic lineage development, and activation-induced cytidine deaminase (AID), a protein that is important in class switch recombination of the immunoglobulin locus, have been shown to be the targets of miR-155.^{16,17} As a consequence of miR-155 downregulation, inefficient germinal center (GC) response causing production of less IgG₁-positive cells was observed. Li *et al*¹⁸ showed that miR-181a can fine-tune T-cell sensitivity during the maturation process by directly targeting SHP-2, PTPN22, DUSP5 and DUSP6, four components of the T-cell receptor signaling pathway. In parallel with the discoveries of important hematopoietic miRNAs, it has become evident that miRNAs are involved in the pathogenesis of leukemia and lymphoma. For example, miR-21, miR-92, miR-150, miR-155 and miR-222 are reported to be deregulated in chronic lymphocytic leukemia (CLL).^{19,20} Overexpression of miR-155 as well as the miR-17-92 cluster has been documented for several B-cell lymphomas, including diffuse large B-cell lymphoma and Hodgkin lymphoma.^{21–23} In a transgenic mouse model, ectopic expression of miR-155 leads to lymphoblastic leukemia/high-grade lymphoma.²⁴

Recently, several studies have been carried out to establish miRNA profiles of hematopoietic cells,^{25,26} but none of them are confirmed by *in situ* hybridization (*ISH*). The aim of our study was to characterize the miRNA expression profile of normal B-cell subsets, which includes naïve, GC and memory B cells. As gene expression analysis identified only limited differences between centroblasts and centrocytes,^{27,28} both cell types were grouped as GC B cells in our study. By comparing these profiles, GC B-cell-specific miRNA expression patterns were defined. Findings from the profiling work were validated with miRNA *ISH* technique and expressions of three miR-150 targets was examined.

MATERIALS AND METHODS

Tissue Sections

Tissue samples of hyperplastic tonsils removed from children with chronic tonsillitis were obtained from the Department of Ear Nose Throat, University Medicine Center Groningen and Academic Medical Center. All the protocols for obtaining and studying human tissues and cells were approved by the institution's review board for human subject research.

FACS-Sorted Normal B-Cell Subsets

Normal B-cell subsets were sorted by FACS from three different tonsil samples, essentially as described earlier.²⁹ Briefly, mononuclear cells were isolated by Ficoll-Isopaque density gradient centrifugation. The collected cell suspension was stained with FITC-conjugated anti-human IgD, PE-conjugated anti-human CD19 (both from Dako, Glostrup, Denmark) and allophycocyanin-conjugated anti-human CD38 (BD Pharmingen, NJ, USA). Naïve B cells (CD19⁺, IgD⁺, CD38⁻), GC B cells (CD19⁺, IgD⁻, CD38⁺) and

memory B cells (CD19⁺, IgD⁻, CD38⁻) were isolated using a FACS aria (BD Biosciences, San Jose, USA). The cells were gated for single cells by forward and sideward scattering. After sorting, the B-cell subsets were lysed in RNA-Bee (Tel-Test Inc. Frindswood, TX, USA) and stored at -80°C, until further processing.

qRT-PCR and Clustering Analysis

The RNA was isolated from normal B-cell subsets with Nucleospin RNA 11 (Macherey-Nagel, Düren, Germany) as described earlier.²⁹ B-cell subset RNA from three different tonsil samples were pooled together accordingly to rule out small differences related to genetic variation in individuals and differences related to histology. We used a commercially available miRNA quantitative RT-PCR (qRT-PCR) profiling kit (Applied Biosystems, Foster City, USA), which, at that time, contained 183 miRNAs. We did not specifically select the miRNAs. qRT-PCR profiling for 183 mature miRNAs were carried out according to manufacturer's protocols. Unsupervised clustering analysis was carried out using *Genesis*.³⁰ A heatmap was generated by mean normalization of experiments and genes, followed by average linkage clustering for all miRNAs that showed a cycle threshold (Ct) value of <35 in at least one of the three B-cell subsets. As the mean and median values for the three B-cell subsets were very similar (Ct value of 31.3–31.9), the mean of all miRNAs assessed was used for normalization ($\Delta Ct = Ct_{miRNA} - Ct_{mean}$). The relative expression levels were determined using the formula $2^{-\Delta Ct}$. Selection of GC B-cell-specific miRNAs was carried out by the following criteria: (1) miRNAs with $2^{-\Delta Ct} > 2$ in at least one of the subsets, that is, miRNAs with a relatively high expression level and (2) miRNA showing at least 4.5-fold difference among the subsets.

miRNA In Situ Hybridization

The miRNA *ISH* was carried out as reported earlier.³¹ Briefly, 11 digoxigenin (DIG)-labeled locked nucleic acid probes antisense to miR-15b, miR-17-5p, miR-21, miR-25, miR-29a, miR-93, miR-106a, miR-146a, miR-122, miR-150 and miR-181b (Exiqon, Vedbaek, Denmark) were used for overnight hybridization on tissue sections at 55°C. Detection was accomplished with anti-DIG alkaline phosphate Fab fragment followed by nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) color development (Roche, Switzerland). miR-122 was chosen as a negative control for staining in tonsil sections, because it has been reported that the expression of miR-122 is detected only in liver.³²

Immunohistochemistry

Slides were deparaffinized and endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 10 min. Antigen retrieval was carried out according to various protocols of the manufacturers. Immunostaining was carried out using antibodies against Survivin (Cell Signaling Technology, Boston,

MA, USA), Foxp1 and c-Myb (Abcam, Cambridge, UK) at a dilution of 1:50–1:400. Signals were amplified by incubation with the appropriate horseradish peroxidase-conjugated antibodies for 60 min and the reactivity was visualized using diaminobenzidine.

Transfection, Western Blotting and DiOC6 Staining

The Epstein-Barr virus negative Burkitt's lymphoma (BL) cell line, DG75,³³ was cultured in RPMI 1640 supplemented with ultraglutamine, 100 U/ml penicillin/streptomycin, and 10% fetal calf serum (Cambrex Biosciences, Walkersville, USA). Transfection of DG75 with synthetic miR-150 mature duplex (Ambion, Austin, USA) was carried out using the A-23 program of the Amaxa nucleofactor I device (Amata, Gaithersburg, USA) with nucleofection solution V. At 24 h post-transfection, half of the cells were lysed, separated in 10% SDS-polyacrylamide gel and immunoblotted for c-Myb, Foxp1 and Survivin with the same antibodies as used for IHC. At 48 h post-transfection, the other half of the cells were incubated with 25 µg/ml DiOC6 at 37°C for 30 min, washed and analyzed by FACS. Cells showing high DiOC6 staining were gated as live cells, whereas cells with low DiOC6 staining were regarded as apoptotic/death cells.

RESULTS

miRNA Profiling For Normal B-Cell Subsets From Tonsils

Out of 183 miRNAs assessed, 86 miRNAs were expressed ($C_t < 35$) in at least one of the three normal B-cell subsets. A heatmap of these 86 miRNAs is given in Figure 1a. To identify miRNAs that are important throughout the GC response, we focused on the miRNAs that showed at least 4.5-fold difference between GC and non-GC B cells, resulting in 16 miRNAs (Figure 1b). A total of 8 of these 16 miRNAs, namely miR-15b, miR-93, miR-181b, miR-25, miR-17-5p, miR-106a, miR-28 and miR-130b showed increased expression levels in GC B cells, whereas miR-150, miR-29a, miR-320 and miR-223 showed a lower expression level in GC B cells. The expression levels of miR-145 and miR-146 increased during the GC transit, whereas the expression levels of miR-331 and let-7a decreased during the GC transit (Figure 1b, Table 1).

miRNA ISH In Tonsils

In view of the differential miRNA expression among the normal B-cell subsets and our main interest in GC B cells, nine miRNAs, which were most abundant and differentially expressed in GC B cells, (the first 9 miRNA listed from the left, Figure 1b) were chosen for miRNA ISH. As the differences in miR-21 expression level among the three B-cell subsets were minimal (~twofold differences, Table 1), this miRNA was chosen as a control for ISH, in which we expect to see no variation in staining intensity within the GC structures. Meanwhile, the liver-specific miR-122 probe was used as the negative control. Out of the 11 miRNAs chosen for ISH, staining for four miRNAs (miR-15b, miR-93, miR-25 and miR-29a) failed in all the cases for unknown reasons.

This failure was not related to the abundance of the miRNAs in GC B cells, as determined by qRT-PCR profiling.

As a negative control, ISH with miR-122, a liver-specific probe, showed negative staining in the tonsil tissue section (Figure 2). miR-21 showed no variation in the staining intensities of GC structures and the mantle zone in tonsil tissue sections as expected. According to morphology and distribution pattern in the tonsil, cells with the strongest miR-21 staining were likely to be interfollicular T cells (Figure 2). The proteinase K step in the ISH procedure precludes double staining of miR-21 and CD3, therefore, we can only suggest that the cells with the strongest miR-21 staining in tonsils represent interfollicular T cells based on their morphology and distribution pattern in the tonsil.

The miRNA ISH results showed weak cytoplasmic staining of miR-150 in GC as compared with the mantle zone, whereas miR-17-5p, miR-106a and miR-181b stained stronger in GC structures (Figure 2). This indicates a higher expression of miR-17-5p, miR-106a and miR-181b and a lower expression of miR-150 in GC B cells as compared with naïve and memory B cells located in the mantle zone (Figure 2). These results were consistent with our miRNA profiling results. For the staining of miR-146a, our miRNA ISH depicted a stronger staining in the GC (Figure 2), mainly contributed by a stronger staining in the centroblasts in the dark zone of the GC, as compared with a weaker signal in the mantle zone (Figure 3). Profiling showed a low expression in naïve cells as compared with GC and memory B cells, which showed an almost similar expression level. As naïve B cells (low miR-146a levels) represent a major population in the mantle zone as compared with the memory cells (high miR-146a levels), this might explain the stronger staining observed in the GC B cell dark zone (Figure 2).

Interestingly, variation in staining intensity was observed within the GC structures for several miRNAs. Gradual decrease of miR-17-5p, miR-106a and miR-181b staining was observed from the dark to the light zone in the GC (Figure 2). miR-150 showed the strongest staining in part of the centroblasts only in the dark zone, whereas the light zone, containing the centrocytes, stained even weaker than the naïve and the memory B cells in the mantle zone (Figure 3).

miR-150 Expression Levels and Its Possible Role In B-Cell Homeostasis

In view of the dynamic expression patterns of miR-150 throughout the GC reaction and its absence in proliferation centers of CLL cases,²⁰ we investigated the possible role of miR-150 in regulation of B-cell homeostasis. One proven target for miR-150, c-Myb,¹⁴ and two putative miR-150 targets, Foxp1 (both PICTAR and TargetScan 4.2 predictions) and Survivin (not predicted by both PICTAR and TargetScan 4.2 programs, but contained miR-150 6mer seed complementary site at its 3' UTR), were examined by immunohistochemistry staining. The c-Myb and Survivin staining patterns were inversely correlated with miR-150 ISH

Figure 1 miRNA profiling of naive, germinal center (GC) and memory B cells. (a) Heatmap of 86 miRNAs, which were expressed ($Ct < 35$) in at least one of the normal B-cell subsets and showed differential expression among the subsets. Green color represents lower Ct value, that is, upregulation, whereas red color represents higher Ct value, that is, downregulation. (b) 16 miRNAs showed at least 4.5-fold difference when compared with GC B cells. The first nine miRNAs and miR-21 (control with not more than twofold changes during the GC transit, not shown in graph) were selected for *ISH*.

staining patterns in tonsils (Figure 4a), supporting the targeting of these genes by miR-150. The staining pattern of Foxp1 did not show a clear inverse pattern with miR-150 *ISH* (Figure 4a). To confirm targeting by miR-150, the BL cell line, DG75, which has a GC B-cell origin and low miR-150 expression level, was transfected with synthetic miR-150 mature duplex for induction of the miRNA (confirmed by

qRT-PCR, data not shown). Western blot analysis of cell lysates showed repression of c-Myb, Survivin as well as Foxp1 in the cells transfected with miR-150 (Figure 4b). These results suggest that the expression of miR-150 either directly or indirectly regulates the expression of c-Myb, Survivin and Foxp1, three genes that are important during B cell development.^{34,35} In addition, DiOC6 analysis for DG75 cell

Table 1 Germinal center-specific miRNAs

	Relative expression, $2^{-\Delta Ct}$			Fold change	
	Naïve B cell	GC B cell	Memory B cell	GC/N	GC/M
<i>Highest in GC</i>					
miR-15b	4.8	31.5	8.9	6.5	3.5
miR-93	2.2	19.6	4.3	8.7	4.5
miR-181b	4.5	16.0	3.1	3.5	5.2
miR-25	2.5	15.7	5.9	6.2	2.7
miR-17-5p	1.0	9.7	3.2	9.8	3.0
miR-106a	0.6	6.0	1.9	10.5	3.1
miR-28	0.3	5.5	0.9	19.9	5.9
miR-130b	0.1	2.6	0.6	21.2	4.0
<i>Lowest in GC</i>					
miR-150	184.1	17.3	160.5	0.1	0.1
miR-29a	30.6	10.3	46.7	0.3	0.2
miR-320	47.2	4.4	11.4	0.1	0.4
miR-223	8.6	0.3	3.5	0.0	0.1
<i>Change during GC transit</i>					
Increase					
miR-145	0.3	3.6	6.8	13.4	0.5
miR-146	0.9	11.7	16.2	12.6	0.7
Decrease					
miR-331	5.8	3.7	0.7	0.6	5.5
let-7a	5.9	0.7	0.8	0.1	0.9
Consistent					
miR-21	7.5	15.1	18.2	2.0	0.8

GC, germinal center. N, naïve B cell. M, memory B cell.

lines 48 h post-transfection with miR-150 showed that there was an increase in the percentage of cells with low DiOC6 staining (Figure 4c). Although the increase was not statistically significant, the trend indicates that the amount of apoptotic/death cells increased upon transfection with synthetic miR-150 mature duplex.

DISCUSSION

Results of miRNA *ISH* on tonsil sections confirmed differential expression of several miRNAs, as identified by the miRNA profiling work. More than 4.5-fold changes in the expression levels of miR-17-5p, miR-106a, miR-146a, miR-150 and miR-181b were found in GC B cells as compared with naïve and memory B cells. A GC B-cell-specific expression pattern was confirmed by miRNA *ISH* staining. To our knowledge, this is the first report confirming distinct miRNA expression patterns in tonsils with *ISH*. Recently, Malumbres *et al*²⁶ did a similar profiling to identify miRNA expression changes during the GC response, but only centroblasts and not centrocytes were used. A total of 39 miRNAs were identified as the classifier to distinguish among T cells, centroblasts, naïve and memory B cells. Our study examined the GC B cells, which included centroblasts and centrocytes, looked for miRNAs with the highest fold change (> 4.5-fold) in naïve or memory compared with GC B cells (T cells not included) and resulted in a total of 16 miRNAs. Despite the differences in cell sorting and profiling technique, we observed a similar expression pattern for 28 out of their 39 classifier miRNAs (Supplementary Data 1). A total of 12 of the 16 miRNAs showing the most extreme changes in the expression levels during GC response in our study are included in their classifier miRNAs. We confirmed differences between GC and naïve/memory B cells for five miRNAs by *ISH* in tonsil tissue sections. We show that *ISH* is a powerful tool to study cell or compartment-specific miRNA expression, which can easily be missed upon analysis from sorted cells.

Figure 2 miRNA expression patterns in tonsils. Tonsil tissue section stained negative when miR-122, a liver-specific probe is used. Staining intensity of miR-21 does not vary between the GC and the mantle zone (MZ), cells with strong positive staining are likely to be interfollicular T cells. miR-150 stains weaker in the GC as compared with the MZ. miR-181b, miR-17-5p and miR-106a stained stronger in the GC than in the MZ, with gradual decrease in the intensity observed from the dark zone to the light zone of the GC. Staining intensity of miR-146a appeared to be stronger in the GC than in the MZ. $\times 10$ magnification is shown for all tonsils.

Figure 3 Close up pictures for miRNA staining patterns seen in germinal center (GC) structures of normal tonsils. Staining intensity of miR-150 was weaker in the GC as compared with the mantle zone (MZ), but some cells in the GC, likely centroblasts, have the strongest staining. Staining intensity of miR-146a appeared to be stronger in the GC, and most cells, which have the strongest staining in the GC, are likely to be centroblasts. The MZ with mixture of naïve and memory B cells stained weaker compared with the GC, presumably because of the domination of naïve B cells (with lower miR-146a expression) in the GC.

Figure 4 miRNA target genes validation. (a) Immunohistochemistry staining showing that the expression of c-Myb ($\times 10$) and Survivin ($\times 20$), but not Foxp1 ($\times 10$), were inversely correlated to miR-150 in tonsils. (b) Downregulation of c-Myb, Survivin and Foxp1 upon miR-150 overexpression in DG75. (c) Although not statistically significant, an increase in the percentage of apoptotic/death cells (low DIOC6 staining) was observed when miR-150 was induced in DG75.

The miRNA *ISH* revealed a distinct staining pattern in the GC structures with decreasing miR17-5p, miR-106a and miR-181b levels from the dark zone to the light zone. This difference was most likely caused by difference in the

expression levels between centroblasts and centrocytes. CD77 is generally accepted as a marker to discriminate between centroblasts and centrocytes.^{36,37} In earlier reported gene expression profiling studies of B cells during GC transit,

Klein *et al*²⁸ found thousands of differences among naïve, GC and memory B cells, but only 19 differentially expressed genes between CD77⁻ and CD77⁺ GC B cells. Consistent with these findings, Hogerkorp and Borrebaeck²⁷ also found no differences in the gene expression profiles between CD77⁻ and CD77⁺ GC B cells. Lack of differences in these studies raised the question whether CD77 can be used as a reliable marker to discriminate between centroblasts and centrocytes. In view of the dynamics of GC B cells to shift from the dark to the light zone,³⁸ and because of the current limitations in isolating pure centroblasts and centrocytes, miRNA *ISH* in normal tissue represents the best tool to study putative differences in miRNA expression levels in these two closely related B cell populations, as differences in *ISH* staining intensities were obviously seen in cells within the GC.

MiR-17-5p and miR-106a are both members of the same seed family, namely the miR-17 family, which also includes miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a and miR-106b. These miRNAs share the same seed sequence and most likely target the same genes, and hence exert similar effects. It has been shown that miRNAs from this miR-17 seed family negatively regulate the expression of cyclin-dependent kinase inhibitor, *CDKN1A* (also known as p21), allowing cells to overcome the G1 cell cycle checkpoint.³⁹ As high expression levels of miR17-5p and miR-106a were observed specifically in centroblasts in the dark zone of GC, whereas *CDKN1A* has been reported to be 30-fold downregulated in centroblasts, we suggest that these miRNAs are essential for centroblasts to progress from G1 to S phase of the cell cycle, by down-regulating *CDKN1A*.^{27,28}

The expression level of miR-146a increases dramatically when the naïve B cells enter the GC and remains high when they exit from the GC. It has been reported that proinflammatory cytokines and microbial components can induce the expression of miR-146a in the acute monocytic leukemia cell line, THP-1.⁴⁰ Luciferase reporter assays showed that miR-146a is a nuclear factor- κ B (NF- κ B)-dependent miRNA.⁴⁰ By targeting IL-1 receptor-associated kinase and TNF receptor-associated factor 6, miR-146a causes a negative feedback for the Toll-like receptor signaling pathway.⁴⁰ As the role of NF- κ B in B-cell homeostasis has been implicated in both immature B cell and also in maintaining GC function,⁴¹ the strong miR-146a staining pattern that we observed in GC correlates with the functional role of NF- κ B in the GC. However, the exact function of miR-146a in this pathway and how it might affect B cell survival should be further investigated.

MiR-181b contains the same seed sequence as miR-181a. MiR-181a has been proven to play a role in hematopoietic differentiation, in favor of B cells¹³ and its expression is regulated during T cell maturation.¹⁸ Recently, it has been shown that AID is a direct target of miR-181b and over-expression of miR-181b in primary splenic B cells reduces the class switch recombination rate.⁴² We observed dynamic expression of both miR-181a and miR-181b during the GC

transit, in which their expression levels peak during the GC stage and fall back to a level similar to that of naïve B cells upon exit from the GC. qRT-PCR profiling revealed a two-fold and a fivefold enrichment in GC B cells for miR-181a and miR-181b, respectively. These results indicate that both miR-181a and miR-181b from the same miRNA seed family are crucial for B cell differentiation and fine-tuning of the GC response.

Our group demonstrated that miR-150 is highly expressed in the majority of CLL tumor cells, but not in the proliferation centers.²⁰ CLL cells are considered to be of memory B-cell origin or derived from naïve B cells.^{43,44} The high miR-150 expression in CLL is consistent with the high miR-150 expression levels that we found in naïve and memory B cells sorted from normal tonsil, and the lack in proliferation centers is consistent with the low levels in GC structures of tonsil. Recently, a key role has been established for miR-150 in B cell development.^{14,15} Overexpression of miR-150 blocks B cell maturation by inhibiting the transition of cells from pro-B to pre-B stage and also induces a slight, but significant, increase in apoptotic rate.¹⁵ Consistent with this finding, Xiao *et al*¹⁴ showed that ectopic miR-150 expression in pro-B cells resulted in an increase in cell death. It was concluded that miR-150 exerts this effect by suppression of its target, c-Myb,¹⁴ a transcription factor which plays an important role during B cell development, maintenance of proliferation as well as cell cycle control of hematopoietic cells.^{35,45–47} c-Myb has already been proven to be important for transition of pro-B to pre-B stage and also for maintenance of follicular B cells, but interestingly, not for mantle zone B cells.³⁵ This is in line with our findings, in which in the mantle zone, the expression of c-Myb indeed was weak, whereas the expression of miR-150 that negatively regulates c-Myb was found to be relatively higher. Similarly, in tonsil sections, Survivin showed an inverse staining pattern with miR-150. For Foxp1, however, such an inverse staining pattern was not obvious, because Foxp1 was also expressed in the mantle zone. Nevertheless, it is still possible that Foxp1 is regulated by miR-150 as Foxp1 knockout mice shared a comparable pro-B to pre-B transition blockade phenotype with mice ectopically expressing miR-150 in hematopoietic cells.^{15,34} According to our Western blotting results, induction of miR-150 resulted in downregulation of c-Myb, Foxp1 and Survivin, supporting the targeting of these genes by miR-150. Nonetheless, we cannot exclude the possibility of an indirect regulatory pathway for the downregulation of Survivin and Foxp1.

It has been reported that c-Myc, which is implicated in the vast majority of BL, can induce the expression of the miR-17-92 cluster and repress the expression of miR-150.^{48,49} In line with the earlier publications,^{14,15} we observed a trend of increased percentage of apoptotic/death cells upon induction of miR-150 in the BL-derived cell line, DG75. Nevertheless, the increased percentage of apoptotic/death cells was not statistically significant. It can be speculated that the induction of

miR-150 was not sufficient to suppress the c-Myc-mediated lymphomagenesis, as the expression levels of the oncogenic miR-17-92 cluster members remains high.

In conclusion, we have determined a miRNA profile of naïve, GC and memory B cells sorted from normal tonsils. miRNAs that were differentially expressed have been identified and verified, for the first time, by miRNA *ISH* in tonsil tissue sections. Remarkably, miRNA *ISH* revealed variation in the expression levels within the GC structures, which indicates the involvement of differentially expressed miRNAs in the functional regulation of centroblasts and centrocytes. Our findings warrant future studies in centroblasts and centrocytes aiming at elucidating the exact pathways targeted by these and possibly by other miRNAs.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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