Identification of microRNAs implicated in the late differentiation stages of normal B cells suggests a central role for miRNA targets ZEB1 and TP53

Giorgio Malpeli1,2, Stefano Barbi2, Simonetta Zupo3, Gabriele Tosadori4, Giovanni Scardoni4, Anna Bertolaso2, Silvia Sartoris5, Stefano Ugel5, Caterina Vicentini2,10, Matteo Fassan6, Annalisa Adamo7, Mauro Krampera7, Maria Teresa Scupoli8, Carlo Maria Croce9, Aldo Scarpa2,10

1Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, Section of Surgery, University of Verona, Verona, Italy
2Department of Diagnostics and Public Health, Section of Pathological Anatomy, University of Verona, Verona, Italy
3Laboratory of Molecular Diagnostics, IRCCS-AOU San Martino-IST, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy
4Center for BioMedical Computing (CBMC), University of Verona, Verona, Italy
5Department of Medicine, Section of Immunology, University of Verona, Verona, Italy
6Department of Medicine, Surgical Pathology and Cytopathology Unit, University of Padua, Padua, Italy
7Department of Medicine, Section of Hematology, Stem Cell Research Laboratory, University of Verona, Italy
8Department of Medicine, Section of Hematology, University of Verona, Italy
9Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA
10Applied Research on Cancer-Network (ARC-NET), University of Verona, Verona, Italy

Correspondence to: Giorgio Malpeli, email: giorgio.malpeli@univr.it

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ABSTRACT

In the late B cell differentiation stages, miRNAs expression modifications promoting or inhibiting key pathways are only partially defined. We isolated 29 CD19+ human B cell samples at different stages of differentiation: B cells from peripheral blood; naïve, germinal center (GC) and subepithelial (SE) B cells from tonsils. SE cells were further split in activated and resting B cell. The miRNA expression profile of these B cells was assessed by microarray analysis and selected miRNAs were validated by quantitative RT-PCR and in situ hybridization on normal tonsils. The comparison of all samples showed changes in 107 miRNAs in total. Among 48 miRNAs differentially expressed in naïve, GC and SE cells, we identified 8 miRNAs: mir-323, mir-138, mir-9*, mir-211, mir-149, mir-373, mir-135a and mir-184, strictly specific to follicular cells that had never been implicated before in late stages of B cell development. Moreover, we unveiled 34 miRNAs able to discriminate between CD5- activated B cells and resting B cell. The miRNAs profile of CD5- resting B cells showed a higher similarity to naïve CD5+ than CD5- activated B cells. Finally, network analysis on shortest paths connecting gene targets suggested ZEB1 and TP53 as key miRNA targets during the follicular differentiation pathway. These data confirm and extend our knowledge on the miRNAs-related regulatory pathways involved in the late B cell maturation.

INTRODUCTION

Newly formed immature B cells, that express an unrefined antigen receptor, are released from the bone marrow to blood circulation and they conclude their differentiation in the follicles of peripheral lymphoid organs [1]. In the immune follicles, a combination of death and survival stimuli results in T-cell dependent selection of functionally mature IgV class-switched and hypermutated antibody-producing B lymphocytes [2]. Ontogenesis of
B cells is accomplished under the concerted action of multiple factors performing transcriptional programs entailing down- or up modulation of genes at each stage of maturation [3, 4]. In this scenario, MicroRNAs (miRNAs) play their own part as well [5].

MiRNAs are non-protein coding RNAs active in the regulation of gene transcription and mRNA translation. MiRNAs action in concert with transcriptional inhibitors and enhancers results in the fine tuning of gene expression. As a consequence, miRNAs influence many cellular processes, such as cell identity, growth, proliferation and control of cell death [6].

Multiple evidences indicated a critical role of specific miRNAs in B cell development [7–9]. In the follicles, during late maturation stages, the down-regulation of mir-150, which controls the transcription factor c-Myb, is requested for the germinal centre (GC) selection and for a correct development of the adaptive humoral immune response [10]. Other miRNAs such as mir-155, mir-181b, mir-15a, mir-16, mir-15b, mir-34a, mir-9, mir-30, let-7a, mir-125b, mir-217 and mir-185 modulate the expression of pivotal genes and functions which contribute to the final B-cell maturation [6]. Also the couple mir-150/c-Myb is involved in the B cell lineage differentiation pathway [11].

The GC is a transient structure that forms within peripheral lymphoid organs in response to B lymphocyte stimulation by T cell-dependent antigens, with the involvement of other immune cells. Up- and down-regulation of multiple miRNAs was detected in B cells at sequential stages of maturation during the GC reaction [12–14]. Some of the miRNAs highlighted by profiling studies were then demonstrated to be functionally relevant [11]. Thus, it appears crucial to identify selective miRNAs that influence and drive B cell development to better characterize the molecular pathways involved in this biological process.

The marginal zone B cells population is heterogeneous, comprising naïve and memory cells [15, 16]. The subepithelial region of tonsils, corresponding to the marginal zone of other peripheral lymphoid organs, on the basis of the pattern of surface markers, contains two subpopulations of supposedly mature CD5 B cells: resting and activated [17]. Activated B cells are IgV gene-hypermutated B cells that achieved the final maturation. Instead, similarly to mice, human SE resting B cells could represent a subpopulation of IgV gene-unmutated B cells survived to the GC selection and waiting for activation by a specific antigen to complete their maturation process [18, 19]. As the miRNAs expression pattern characterizes different B cell maturation stages, it could also contribute to define the ontogenesis of these two B cell subsets.

In this study, we compared the expression profiles of miRNAs in CD19+ B cells at different late differentiation steps by microarray analysis. In addition, we compared the expression profile of miRNAs in subepithelial activated and resting B cells, to better define the identity of the two B cell subtypes. Experimentally validated gene targets of differentially expressed miRNAs were subjected to network analysis, to infer the underlying regulatory pathways. By this procedure, we tried to identify protein hubs and cell functions operated by miRNAs.

RESULTS

One hundred and seven single miRNAs were significantly differentially expressed in CD19+ B cells from blood compared to B cells from tonsils at different stages of activation

We assessed miRNAs expression in B cell subsets representing stages of development ranging from CD19+ peripheral blood B cells to mature B cells in the immune follicles of tonsils. We analyzed a total of 29 B cell samples by microarray technology: four samples of CD19+ B cells isolated from peripheral blood; 14 B cell samples isolated from tonsil folicles, that were divided in three different groups: naïve CD5− B cells (2 samples), CD23 CD39+ GC B cells (12 samples) and CD5− SE mature B cells (11 samples). Finally, among the 11 samples of mature CD5− B cells, we defined two distinctive subgroups: the IgV-hypermutated activated B cells (4 samples) and the IgV-non-hypermutated resting B-cells (seven samples). On summary, on the basis of the differentiation stage, our B cell samples could be divided in four different experimental groups, including the subepithelial CD5− B cells which are subdivided in two subgroups.

The heat map in Supplementary Figure 1 shows the levels of differentially expressed miRNAs among four B-cell subsets. The hierarchical clustering emphasized the correlation among samples belonging to the same differentiation stage (Supplementary Figure 1A). One hundred and seven single miRNAs were differentially expressed among the five B-cell subsets at FDR 1% (Supplementary Figure 1B). The list of 135 probes representing 107 single miRNAs is reported in Supplementary Table 1.

Most of miRNA expression differences occurred between CD19+ and naïve CD5− cells. Among follicular B cell populations, CD23 CD39+ GC B-cells showed an expression profile well distinct from that of naïve CD5− B cells and mature CD5− B cells. Indeed, these latter two cell populations showed only a limited number of differentially expressed miRNAs. Therefore, this analysis highlighted the strong modulation of miRNA expression during the late B cell differentiation.

The expression of thirty-seven single miRNAs distinguishes peripheral blood CD19+ from CD19+/CD5− B cells of lymph node

About 70% of peripheral blood CD19+ cells are immature or naïve CD19+/CD5− B lymphocytes before joining peripheral lymphoid compartments for the
The average expression levels of the top 20 significantly differentially expressed miRNAs (Figure 2) demonstrated that they were mostly upregulated in mature B cells. In contrast, miR-150, miR-361, miR-221, miR-135a, miR-141, miR-185 and miR-149 decreased in GC B cells compared to naïve B cells.

Finally, naïve CD5+ B-cells shared with activated CD5+ B-cells a specific group of miRNAs whose expression resulted downregulated in CD23 CD39+ B-cells (Figure 1). In addition, among miRNAs expressed at higher level in CD5+ B cells compared to CD5+ B cells, we identified five miRNAs: miR-29a, miR-29b, miR-29c, miR-26a and miR-221. The expression level of these miRNAs was comparable to those of CD23 CD39+ B cells. Therefore, we can conclude that the B cell maturation step allowing the entrance in peripheral lymphoid organs induces the upregulation of a restricted group of miRNAs: miR-29a, miR-29b, miR-29c, miR-26a and miR-221.

To better reinforce our original data, by comparing our results with published data we generated a list of 48 differentially expressed miRNAs [12–14] (Table 2). The four studies considered for the comparison, including the present study, demonstrated the higher expression in naïve B-cells of mir-320, the up-regulation of mir-181b, mir-25, mir-130b in GC B cells as well as the greater expression of both mir-29a and seven members linked to the cluster mir-17/92 in mature B cells. Moreover, in at least one of the four studies, 35 of 48 differentially expressed miRNAs were expressed at higher level in different B cell subsets; on the contrary, 27 miRNAs were not differentially expressed or not detected. However the four studies presented a controversial expression of mir-185: we detected levels of mir-185 higher in naïve than in GC-restricted B cells (Figure 1), whilst both Malumbres et al. [12] and Belver et al. [21] showed mir-185 upregulation in GC B cells.

Our study identified 8 new differentially expressed miRNAs: mir-323, mir-138, mir-9*, mir-211, mir-149, mir-373, mir-135a and mir-184; that have not been reported in literature so far.

Expression of thirty-three selective miRNAs discriminates subepithelial resting and marginal zone CD5+ activated B-cells

As described by Dono M et al., 2000 [17], resting CD5+ B cells were identified in the SE region of tonsils as small size CD5+ B cells expressing unmutated IgV genes. The ontogenesis of this cell population has not been defined yet, as well as the difference between resting and activated B cells, generated from B cells of GCs adjacent to SE. Therefore, we proceeded to analyze the miRNAs profiles of these two B cell subtypes, comparing four samples of CD5+ activated B cells to seven samples of CD5+ resting B cells (Figure 3). The two cell populations clustered separately and they are strongly distinguishable
for the differential expression of 34 miRNAs (FDR 5%). The miRNAs profile comparison between resting and activated B cells showed the up-regulation of 19 miRNA in activated B cells: mir-98, mir-106a, mir-20a, mir-17-5p, mir-20b, mir-16-2, mir-18a, mir-155, mir-21, mir-181d, mir-425-5p, mir-148a, mir-15b, mir-15a, mir-181b mir-181c, mir-181a, mir-130b, mir-148b (Table 3). Conversely, 15 miRNAs resulted downregulated in activated B cells: mir-483, mir-95, mir-326, mir-135a, mir-184, mir-185, mir-516-3p, mir-30b, mir-203, mir-216, mir-150, mir-182*, mir-141 and mir-211 (Table 3).

Validation of miRNAs expression by quantitative RT-PCR

We validated our microrray results by quantitative RT-PCR on CD5+ GC and CD5− activated and resting B cell mRNA samples as shown in Supplementary Figure 3. In fact, we validated 10 different miRNAs: mir-150, mir-20b, mir-23a, mir-211, mir-15b, mir-21, mir-106a, mir-146a, mir-9* and mir-155 whose expression trends by quantitative RT-PCR highlighted the same expression trend shown by microarray analysis. The only discrepancy between RT-PCR and microarray analysis data was referred to miR-23a expression: this miRNA, in fact, did not show a significantly differential expression among the four B cell subsets by microarray analysis but it did show a significant upregulation by RT-PCR (P = 0.002) in CD5− activated B cells compared to the other B cell subsets. Statistical analysis of quantitative RT-PCR results using Kruskal-Wallis test confirmed the differential expression of these miRNAs among the B cells subsets analyzed (Supplementary Figure 3).

Validation of miR-9*, miR-29b and miR-150 on normal tonsils by in situ hybridization

We validated by in situ hybridization miR-9*, miR-29b and miR-150, selected from microrray results. The analysis was conducted in five pharyngeal tonsils.

Figure 1: Expression profile of miRNAs in cell subsets representing different stages of B cell maturation. CD5+: naïve B cells from tonsils; CD23−CD39+: germinal centre (GC) B cells from tonsils; CD5−: subepithelial (SE) mature B cells from tonsils, subdivided in CD5− resting and CD5− activated B cells. (A) Array tree of 25 samples representing different stages of maturation of the B cells based on the expression levels of miRNAs. (B) The heat map describes the expression levels of 48 differentially expressed miRNAs in 25 samples owning to four B cell subsets isolated from tonsils (FDR 1%). Clusters 1, 2 and 3 are three miRNAs groups isolated by clustering procedures. Red, higher expression (log₂, +4); green, lower expression (log₂, −4).
using mature miRNA labeled probes (Figure 4). The expression of the three miRNAs was detectable as a grainy blue cytoplasmic staining. MiR-9* was significantly overexpressed in germinal centers (GC), mantle zone (MZ), and subepithelial marginal zone (MaZ) in comparison to squamous epithelium (Sq). At higher magnification, it is evident a stronger expression in GC cells in comparison to MZ. MiR-29b was significantly overexpressed in both MZ and MaZ in comparison to GC. MiR-150 was significantly downregulated in GC in comparison to both MZ and MaZ.

Identification of genes regulated by differentially expressed miRNAs in the late B cell development

Using the differentially expressed miRNA profiles, we generated a list of 608 experimentally validated gene targets (Supplementary Table 3), that were filtered through the gene ontology category “lymphocyte differentiation” (GO:0030098). The distribution of 28 miRNA targets generated by the three clusters of differentially expressed miRNAs is shown in Figure 5A, where the Venn diagram highlighted two shared selective gene targets among the three miRNA clusters: ZEB1 and TP53 genes. In particular, we identified two selective miRNA lists: the first one, composed by miR-150, miR-130b, miR-141, miR-29b, miR-26a, miR-34a and miR-200c; able to target the ZEB1 gene; and the second one, composed by miR-150, miR-221, miR-21 and miR-25, able to target the TP53 gene. The only common miRNA between the two lists is miR-150.

Finally, we compared the expression levels of seven ZEB1- and four TP53-targeting miRNAs in naïve and GC B cells (Figure 5B): all ZEB1-targeting miRNAs were expressed at higher level in naïve compared to GC B cells; while only three (miR-221, miR-21 and miR-25) out

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**Figure 2:** Expression levels of top 30 differentially expressed miRNAs in cell subsets representing different stages of late B cell differentiation. Graphs report expression levels of 30 miRNAs with lower Q value among the 48 differentially expressed miRNAs in 29 samples divided in four B cell subsets. Color graphs indicate the miRNAs included in Cluster 1 (yellow), Cluster 2 (orange) and Cluster 3 (green). Data were obtained from microrray analysis after normalization and log₂ transformation of the probe signal. 0: naïve B cells; 1: germinal centre B cells; 2: subepithelial activated B cells; 3: subepithelial resting B cells.
Table 1: List of differentially expressed miRNAs among CD5⁺ B cells, CD23⁻/CD39⁻ B cells and CD5⁻ B cells (FDR 2%)

| miRNA     | Q value |
|-----------|---------|
| miR-150   | 0.0077  |
| miR-17-5p | 0.0077  |
| miR-106a  | 0.0077  |
| miR-20a   | 0.0077  |
| miR-181b  | 0.0077  |
| miR-361   | 0.0077  |
| miR-130b  | 0.0077  |
| miR-20b   | 0.0077  |
| miR-18a   | 0.0081  |
| miR-29b   | 0.0082  |
| miR-148a  | 0.0082  |
| miR-191   | 0.0083  |
| miR-93    | 0.0086  |
| miR-26a   | 0.0086  |
| miR-221   | 0.0086  |
| miR-155   | 0.0087  |
| mir-15b   | 0.0087  |
| miR-29a   | 0.0087  |
| miR-138   | 0.0098  |
| miR-106b  | 0.0102  |
| miR-135a  | 0.0102  |
| miR-21    | 0.0102  |
| mir-16-2  | 0.0102  |
| miR-181c  | 0.0102  |
| miR-141   | 0.0102  |
| mir-185   | 0.0102  |
| miR-149   | 0.0106  |
| miR-181d  | 0.0110  |
| miR-425-5p| 0.0110  |
| miR-29c   | 0.0110  |
| miR-181a  | 0.0110  |
| miR-25    | 0.0124  |
| mir-204   | 0.0136  |
| mir-184   | 0.0136  |
| miR-148b  | 0.0136  |
| miR-373   | 0.0138  |
| miR-9*    | 0.0138  |
| mir-30b   | 0.0138  |
| miR-19b   | 0.0147  |
| mir-320   | 0.0153  |
| miR-146a  | 0.0153  |
| miR-30a-3p| 0.0156  |
| miR-9     | 0.0156  |
| mir-323   | 0.0159  |
| mir-211   | 0.0159  |
| miR-328   | 0.0164  |
| mir-335   | 0.0178  |
| miR-15a   | 0.0198  |
Table 2: B cell subsets with highest level of miRNAs significantly modulated during the late differentiation of B cells: a comparison with literature data

| miRNA       | Malpeli G et al. | Zhang J et al. | Malubres S et al. | Tan LP et al. |
|-------------|------------------|----------------|-------------------|---------------|
| miR-323     | GC               | ND             | ND                | ND            |
| miR-138     | GC               | ND             | ND                | ND            |
| miR-204     | GC               | GC             | ND                | ND            |
| miR-181b    | GC               | GC             | GC                | GC            |
| miR-25      | GC               | GC             | GC                | GC            |
| miR-9*      | GC               | ND             | ND                | ND            |
| miR-16-2    | GC               | ND             | GC                | GC            |
| miR-15a     | GC               | GC             | ND                | ND            |
| miR-15b     | GC               | GC             | GC                | ND            |
| miR-425-5p  | GC               | ND             | ND                | GC            |
| miR-148a    | GC               | Mat            | GC                | GC            |
| miR-148b    | GC               | ND             | GC                | Naïve         |
| miR-155     | GC               | ND             | GC                | ND            |
| miR-146a    | GC               | GC-Mat         | Mat              | ND            |
| miR-19b     | GC               | GC             | GC                | GC            |
| miR-130b    | GC               | GC             | GC                | GC            |
| miR-93      | GC               | GC             | GC                | GC            |
| miR-106b    | GC               | GC             | GC                | GC            |
| miR-18a     | GC               | GC             | GC                | GC            |
| miR-17-5p   | GC               | GC             | GC                | GC            |
| miR-20b     | GC               | GC             | GC                | GC            |
| miR-106a    | GC               | GC             | GC                | GC            |
| miR-20a     | GC               | GC             | GC                | GC            |
| miR-181a    | GC               | ND             | GC                | GC            |
| miR-181c    | GC               | ND             | Naïve-GC          | ND            |
| miR-181d    | GC               | GC             | Naïve-GC          | ND            |
| miR-191     | GC               | ND             | GC                | GC            |
| miR-21      | Mem              | Mem            | GC                | Mem           |
| miR-30a-3p  | Mem              | GC             | GC                | ND            |
| miR-361     | Mem              | Mem            | ND                | GC            |
| miR-29c     | Mem              | Mem-Naïve      | GC                | Mem           |
| miR-29b     | Mem              | ND             | GC                | Mem-Naïve     |
| miR-29a     | Mem              | Mem-Naïve      | Mem              | Mem           |
| miR-26a     | Mem              | Naïve          | Naïve             | Mem           |
| miR-221     | Mem              | Mem            | Naïve             | Naïve-Mem     |
| miR-150     | Naïve            | ND             | Mem              | Naïve         |
| miR-9       | Naïve            | GC             | ND                | ND            |
| miR-30b     | Naïve            | GC             | Mem              | GC            |
| miR-141     | Naïve            | Naïve          | ND                | ND            |
| miR-211     | Naïve            | ND             | ND                | ND            |
| miR-149     | Naïve            | ND             | ND                | ND            |
| miR-373     | Naïve            | ND             | ND                | ND            |
| miR-135a    | Naïve            | ND             | ND                | ND            |
| miR-320     | Naïve            | Naïve-Mem      | Naïve-Mem         | Naïve-Mem     |
| miR-335     | Naïve            | Naïve          | ND                | ND            |
| miR-185     | Naïve            | ND             | GC                | ND            |
| miR-184     | Naïve            | ND             | ND                | ND            |
| miR-328     | Naïve            | ND             | GC-Mem            | ND            |

ND: not detected or not differentially expressed; Naïve: naïve B cells; GC: germinal centre B cells; Mat: mature B cells. Zhang J et al. [14], Malubres S et al. [12], Tan LP et al. [13].
of four TP53-targeting miRNAs (except miR-150) were upregulated in GC B cells.

**Network analysis based on the experimentally validated gene targets of differentially expressed miRNA**

To discover the selective cellular pathways targeted by the identified miRNAs, we performed a network analysis. To infer proteins and cellular pathways modulated by miRNAs, we consulted a global intracellular protein-protein interactome (http://dp.univr.it/~laudanna/LCTST/downloads/index.html). For each cluster of differentially expressed miRNAs, we identified the nodes involved in the shortest paths among experimentally validated miRNA targets listed in Figure 5A. We considered the proteins with highest degree (hubs) as the most perturbed targets by the biological processes under investigation. Finally, we generated for each miRNA cluster a sub-network including only the hubs and the

**Figure 3: Differential expression of miRNAs in subepithelial CD5− activated and resting B cell subsets.** The heat map reports the expression levels of differentially expressed miRNAs between two subepithelial (SE) CD5− B cell populations (FDR 10%): activated IgV mutated SE B cells and resting IgV unmutated SE B cells. Red, higher expression (log2, +4); green, lower expression (log2, −4).
paths that are connected to the differentially expressed miRNAs. Using this representation, we highlighted the relationship between miRNA-related changes and potential protein targets. We identified a cluster 1-related sub-network that was composed by 133 items, including the 5 main hubs (APC, ATM, CTNNB1, ERBB2, TP53); a cluster 2-related sub-network that contained 30 items, including the 6 hubs (GRB2, HDAC4, HSP90AA1, PIK3R1, TP53 and ZEB1) and, finally, a cluster 3-related sub-network that included 9 hubs (APC, BCL6, ELAVL1, HDAC1, HDAC2, HSP90AA1, SOCS5, SUMO1 and ZEB1) (Figure 6A–6C respectively).

**Gene ontology analysis**

To better define the miRNA-related regulatory potential functions on controlling B cell biological modifications during the GC reaction, we analyzed

### Table 3: List of differentially expressed miRNAs between subepithelial CD5− activated and resting B cells (FDR 10%)

| miRNA  | *activated vs resting | Q value |
|--------|-----------------------|---------|
| mir-211 | Down                  | 0.0026  |
| miR-148a | Up                    | 0.0340  |
| mir-141 | Down                  | 0.0445  |
| miR-155 | Up                    | 0.0445  |
| mir-184 | Down                  | 0.0445  |
| miR-15b | Up                    | 0.0445  |
| mir-30b | Down                  | 0.0445  |
| miR-150 | Down                  | 0.0445  |
| miR-148b | Up                  | 0.0445  |
| mir-16-2 | Up                    | 0.0445  |
| miR-216 | Down                  | 0.0445  |
| mir-185 | Down                  | 0.0445  |
| miR-18a | Up                    | 0.0445  |
| miR-17-5p | Up                   | 0.0448  |
| miR-130b | Up                   | 0.0448  |
| miR-516-3p | Down              | 0.0448  |
| miR-106a | Up                   | 0.0500  |
| miR-21 | Up                    | 0.0500  |
| miR-95 | Down                  | 0.0551  |
| miR-20b | Up                    | 0.0833  |
| miR-425-5p | Up                | 0.0833  |
| miR-181b | Up                   | 0.0833  |
| miR-181a | Up                   | 0.0833  |
| miR-135a | Down                 | 0.0833  |
| miR-181c | Up                   | 0.0853  |
| mir-203 | Down                  | 0.0855  |
| miR-15a | Up                    | 0.0855  |
| miR-483 | Down                  | 0.0855  |
| miR-182* | Down                | 0.0855  |
| miR-20a | Up                    | 0.0855  |
| miR-181d | Up                   | 0.0909  |
| miR-98 | Up                    | 0.0911  |
| mir-326 | Down                  | 0.0986  |

*Up: higher expression in activated CD5− B cell.
Down: higher expression in resting CD5− B cell.
the gene annotation of all primary protein-interactors connected to the shortest paths analyses. We identified 52 Panther pathways and 49 Panther hallmarks that were significantly over-represented as reported in Supplementary Table 4 and Supplementary Table 5, respectively. The top 10 enriched cellular pathways were: “CCKR signaling map” \((P = 1.66 \times 10^{-44})\); “Gonadotropin releasing hormone receptor pathway” \((P = 1.44 \times 10^{-44})\); “Angiogenesis” \((P = 7.15 \times 10^{-39})\); “EGF receptor signaling pathway” \((P = 7.92 \times 10^{-36})\); “Apoptosis signaling pathway” \((P = 5.86 \times 10^{-33})\); “Interleukin signaling pathway” \((P = 6.91 \times 10^{-35})\); “B cell activation” \((P = 1.73 \times 10^{-32})\); “T cell activation” \((P = 1.14 \times 10^{-30})\); “Inflammation mediated by chemokine and cytokine signaling pathway” \((P = 8.44 \times 10^{-28})\); “Integrin signalling pathway” \((P = 1.04 \times 10^{-27})\) (Supplementary Table 4).

The top 10 enriched hallmarks categories were: “Genes up-regulated during transplant rejection” \((P = 7.09 \times 10^{-37})\); “Genes up-regulated by activation of the PI3K/AKT/mTOR pathway” \((P = 7.47 \times 10^{-46})\); “Genes involved in the G2/M checkpoint, as in progression through the cell division cycle” \((P = 7.91 \times 10^{-46})\); “Genes important for mitotic spindle assembly” \((P = 1.66 \times 10^{-43})\); “A subcluster of genes regulated by MYC - version 1 (v1)” \((P = 12.14 \times 10^{-42})\); “Genes encoding cell cycle related targets of E2F transcription factors” \((P = 1.85 \times 10^{-35})\); “Genes mediating programmed cell death (apoptosis) by activation of caspases” \((P = 3.82 \times 10^{-31})\); “Genes regulated by NF-kB in response to TNF [GeneID = 7124]”; \((P = 3.87 \times 10^{-31})\); “Genes involved in p53 pathways and networks” \((P = 4.87 \times 10^{-29})\); “Genes up-regulated in response to IFNG [GeneID = 3458]” \((P = 5.00 \times 10^{-23})\) (Supplementary Table 5).

**DISCUSSION**

MiRNAs establish regulatory networks by intersecting and affecting multiple signaling pathways [22, 23]. Trying to disclose the miRNA-related regulatory actions during the late phase of B cell differentiation, we firstly identified miRNAs profiles of B cell subsets in different maturative phases and their prospective relationships in association to the cell machinery. Using this strategy, we identified molecular pathways that are modulated by selective miRNAs.

Microarray or quantitative RT-PCR analyses have already assessed the miRNAs involved in the late differentiation of B cells [12–14]. The results reported in literature, however, are partially conflicting about both the number of differentially expressed miRNAs and their expression levels. In this complex scenario our data achieved some essential goals that can be summarized in the following points: 1) the cell transition from immature to mature B lymphocytes is strictly characterized by the modulation of 107 miRNAs: 33 miRNAs change their expression during B cell passage from blood to peripheral lymphoid organs; 2) the three main B cell populations in tonsils (naïve, GC and SE B cells) involved in the GC

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**Figure 4: MiR-9*, miR-29b and miR-150 distribution in normal tonsillar tissue.** The expression of the three miRNAs was detectable in pharyngeal tonsils as a grainy blue cytoplasmic staining. **MiR-9** was significantly overexpressed in germinal centers (GC), mantle zone (MZ), and subepithelial marginal zone (MaZ) in comparison to squamous epithelium (Sq). At higher magnification, it is evident a stronger expression in GC cells in comparison to MZ. **MiR-29b** was significantly overexpressed in both MZ and MaZ in comparison to GC, whereas **miR-150** was significantly downregulated in GC in comparison to MZ and MaZ. Bar scale = 200 µm.
reaction showed distinct expression profiles: 48 miRNAs are differentially expressed during the GC reaction and they are segregated in three clusters according to the sequential maturative stages; 3) among the 48 differentially expressed miRNAs, we identified 8 new original miRNAs (mir-323, mir-138, mir-9*, mir-211, mir-149, mir-373, mir-135a and mir-184) that were not previously reported as discriminative miRNAs during B cell development; 4) the expression of 34 miRNAs are able to discriminate between resting and activated CD5− SE B cells; moreover, miRNAs from resting CD5− B cell better resemble miRNAs from naïve B cell rather than miRNAs from activated CD5+ B cell; 5) during the late phase of B cell differentiation ZEB1 and TP53 are the most influenced targets by the differentially expressed miRNAs; 6) APC, ATM, CTNNB1, ERBB2, TP53, BCL6, SOCS5, HDAC4, HDAC2, HDAC1, GRB2, PIK3R1, HSP90AA1, ZEB1, SUMO1 and ELAVL1 are the main hub proteins that are controlled by the identified miRNAs; 7) gene ontology analysis showed that miRNAs controls core signaling pathways and essential for B cell functions.

Moreover, our new identified differentially expressed miRNAs are related to other specific aspects of B cell biology. We discovered that miR-323 expression was reduced in activated mature B cells compared to naïve and GC B cells. This miRNA was reported as a biomarker of immune and inflammatory response, since it is an activator of the Wnt/Cadherin signaling pathway and it induces cytokine expression and tissue metalloproteases [24] in rheumatoid arthritis. Moreover, miR-323 was recently described to target EED, a component of the polycomb repressive complex 2 (PRC2) [25], that plays an essential role in GC formation; in fact, the modulation of miR-323 in the follicles was reported as a critical and relevant event.

**Figure 5:** (A) Distribution 28 genes targeted by differentially expressed miRNAs in the three clusters reported in Figure 1. Venn diagram reports the distribution and intersection of target genes of differentially expressed miRNAs which belong to the gene ontology category “lymphocyte differentiation”. Two boxes indicate the miRNAs known to target ZEB1 and TP53. (B) Differential expression of miRNAs known to target ZEB1 and TP53 between naïve and GC B cells.
of the adaptive immune response [26]. Recently, *mir-138* was shown to target acyl protein thioesterases (*LYPLA1* and *LYPLA2*) able to regulate the palmitoylation of CD95 and apoptosis in chronic lymphoblastic leukemia [27]. *Mir-138* was found upregulated in follicular lymphoma but downregulated in the GC subtype of diffuse large B cell lymphoma [28] [29]. The over-expression of *mir-211* has been already associated with lymphoma progression [30]. By contrast, specific alterations of *Mir-9* expression were never reported in normal B cell populations, even if this miRNA is a selective marker of follicular lymphomas [28]. Similarly, the differential expression levels of *mir-149* were never described during physiological B cell development even if its downregulation have been associated to both diffuse large and follicular lymphoma [28, 31]. Finally, the differential expression of *mir-373* was reported in several neoplastic diseases except in lymphomas [32]; on the contrary, both the *mir-135a* downregulation (able to target *JAK2*) and *miR-135a* overexpression were associated to relapse of Hodgkin’s [33] and follicular and diffuse large B cell lymphomas [29, 31]. Finally, the downregulation of *mir-184* was reported in marginal zone lymphoma [34].

A very interesting observation is related to *miR-185* expression pattern: published results, in fact, demonstrated that this miRNA was upregulated in mouse GC B cells and downregulated in human GC B cells after stimulation of naïve B cells with lipopolysaccharide antigen [21, 35]. *MiR-185* is able to switch off the *BTK* kinase, that plays a critical role in controlling the positive and negative selection of autoreactive B cells in mice [36], by influencing the B cell receptor signaling pathway [21]. Our data confirmed that *miR-185* is downregulated in human GC B cells. In fact, based on these evidences, we speculate that *miR-185* level, in connection with other regulatory mechanisms having different weights in human and mice, participates to the fine tuning of the threshold for the selection of autoreactive B cells.

Figure 6: Network of proteins obtained by shortest path analysis among target genes of miRNA differentially expressed (Figure 1), their first interactor proteins and miRNAs targeting hub proteins and first interactors. Dark grey square: hub proteins, that is proteins nodes of the network with higher degree; light grey circle: protein first interactors of hub proteins; white hexagon: miRNA targeting hub proteins. In tables, list of hub proteins (bold letters), first interactors of hub proteins and miRNAs targeting hub proteins and their first interactors present in the network. (A) Network of target proteins of miRNA listed in Cluster 1 of Figure 1, their first interactor proteins and miRNAs targeting hub proteins. (B) Same representation as in 6A by using the miRNAs listed in Cluster 2 of Figure 1. (C) Same representation as in 6A by using the miRNAs listed in Cluster 3 of Figure 1.
The SE niche of tonsils is populated by mature CD5− B cells comprising small resting B lymphocytes and activated plasmablasts with peculiar phenotypic characteristics [37, 38]. According to the classical view of the primary immune response, the non-cycling hypermutated class-switched mature B cells are the progeny of adjacent positively antigen-selected GC B cells [17, 39]. Instead, the nature of SE resting cells not-class switched, IgV gene unmutated and insensitive to T helper-mediated activation, remains elusive [17, 40, 41]. In mice, the memory pool contains stable fractions of unmutated and mutated IgV gene cells [19]. In humans, the memory B cell compartment is not totally well defined and the existence of a T helper- and GC-independent pathway of maturation of B cells is still controversial [41]. According to our data, activated and resting mature B cells have unique expression profiles of miRNAs. These data reinforced the hypothesis that these two B cell populations are truly two distinct entities: in fact, the miRNA profile of resting CD5− B cells better resembled that of naive CD5+ B cells rather than that of activated CD5+ B cells. In addition, the SE resting cells showed the upregulation of a set of miRNAs typically upregulated in activated mature B cells compared to GC B cells. Therefore, our results clearly demonstrated that resting CD5− B cells could be defined as a specific subpopulation of CD5− B cells that have exceed the GC selection and partially acquired the features of mature B cells.

In the GC reaction, the balance between positive and negative inputs is critical for an effective physiological response to antigens [42]. We identified the ZEB1 and TP53 genes as main targets of differentially expressed miRNAs during the late B cell differentiation. ZEB1–targeting miRNAs are downregulated on GC B cells, suggesting a possible biological effect on B cells promoted by the increased ZEB1 expression level. ZEB1 is involved both in cell reprogramming and in the epithelial-to-mesenchymal transition; but its role in B cell development is still unclear. In mice, Zeb1 expression is required for GC formation and memory B cell response. The enforced Zeb1 expression after the B cell receptor (BCR)-antigen binding promoted both proliferation and altered apoptotic death of naïve B cells [43]. Moreover, Zeb1 plays a critical role in regulating the functions of BCL6 that is a rheostat factor in controlling B cell fate. In fact, ZEB1 binds the E-box motif that controls the BCL6 and SMAD genes regulating the GC reaction and the TGF-β1 signaling pathway, respectively [44]. In humans, Zeb1 is almost absent in quiescent B cells of the mantle zone but it is expressed in proliferating GC B cells of tonsils (http://www.proteinatlas.org/ENSG00000148516-ZEB1/tissue/tonsil). High Zeb1 expression was also associated with adverse overall survival in diffuse large and mantle B cell lymphomas [45–47]. The higher Zeb1 expression levels in malignant lymphomas suggest its involvement during B cell differentiation either at early stages or after BCR activation. Therefore, all these evidences indicate that miRNAs could contribute to set Zeb1 levels and action timing during early cellular events following BCR activation.

TP53 exerts a central role in maintaining cell homeostasis following genotoxic insults. BCL6 modulates the B cell response inducing tolerance to DNA damage-induced apoptosis by suppressing TP53 in GC B cells, [48]; while P53 controls the cell cycle at two distinctive checkpoints (G1/S and G2/M) by the regulation of miR-107, miR-145, miR-34, and of the miRNA clusters miR-15a/miR-16 and miR-192/miR-194/miR-215, able to target many cell cycle-related genes [49]. An increased level of TP53-targeting miRNAs in GC B cells such as miR-21, miR-25 and miR-200c could promote an additional control level of TP53 expression after the activation of the DNA damage response.

By expanding our analysis through the protein interactome, we identified hub proteins under the control of modulated miRNAs during the late B cell differentiation. These target proteins are involved in different pathways suggesting that these miRNAs can regulate several signaling networks that are activated during B cell development. We identified some hub proteins that have been already reported as key player in main signaling pathways, but also some specific hubs that were recently directly implicated in the B cell development. In particular, we identified ELAVL1 (also known as HuR), that plays an essential role in the GC reaction and class-switched antibodies production [50]. We demonstrated that miR-9, which targets ELAVL1, decreased its expression in the transition from naïve to GC and mature B cells, suggesting that miRNA could control HuR expression levels.

In conclusion, we described the modulation of miRNAs and the involvement of underlying miRNA targets and cellular functions in the late B cell maturation. Our findings complement the results reported in previous studies and offer new hints for further functional studies addressing the comprehension of the role played by miRNAs in B lymphocyte biology.

MATERIALS AND METHODS

Samples

Twenty-nine CD19+ normal B cell samples were studied. Four CD19+ B cell samples were obtained from the peripheral blood of four healthy donors and twenty-five CD19+ normal B cell samples were prepared from nonpathological tonsils after surgical removal at the National Institute for Cancer Research of Genova. The materials used have been collected under Program 1-IC01 protocol IC/01/LLC/001 13/12/2001, renewed 28/11/2013. The protocols, which concerned the collection of the informed consent of patients, were approved by the local
ethics committee of the IRCCS Azienda Ospedaliera Integrata San Martino – IST Istituto Nazionale per la Ricerca sul Cancro (Genova). Among B cell samples from tonsils, two were naïve CD5+ B cells (each sample was a pool of five different donors), twelve samples were CD23-CD39- GC B cells; eleven samples were CD5- subepithelial mature B cells. Among the eleven SE CD5- B cell samples, four samples were SE CD5- IgV hypermutated activated B cells and seven samples were SE CD5- not IgV hypermutated resting B cells. Subepithelial CD5- B cells of tonsils are equivalent to the marginal zone B cells in other peripheral lymphoid organs [17].

Cell separations and RNA extraction

Pure populations of B lymphocytes were isolated by antibody-conjugated magnetic beads sorting according to established protocols. CD19+ cells were isolated from the peripheral blood of healthy donors by positive selection with anti-CD19 antibody magnetic beads-conjugated and magnetic sorting. Four CD19+ B cell populations, naïve B cells (CD5+), GC B cells (CD23-CD39+) and two populations of CD5- B cells, SE activated IgD+ (IgV gene mutated) and SE resting IgD+ (IgV gene unmutated), were isolated from tonsils as previously described [17]. Purity of B lymphocytes was at least 95%, according to cytfluorometer analysis. Total RNA was isolated from cells using TRIzol reagent (Invitrogen) according to manufacturer protocol. RNA concentration and integrity was determined respectively by spectrophotometer and agarose gel separation.

Microarrays

MicroRNA labeling and hybridization were performed using 5 mg micrograms total RNA, as described [51]. We used a multi-species microarray platform containing 2284 probes, 1256 for human and 1028 for mouse targets, respectively. A total of 353 human mature miRNA containing 2284 probes, 1256 for human and 1028 for mouse targets, respectively. A total of 353 human mature miRNA were detected with Streptavidin-Alexa647 conjugate and transformed using the medpolish algorithm, in order to obtain a unique expression figure for each target. Clustering analysis was performed using the hclust function and the inverse Pearson correlation as a distance metric, for both genes and arrays. All the clusters were visualized using the Java TreeView software (http://jtreeview.sourceforge.net). To select differentially expressed genes, we performed either anova or t-tests. To take into account multiple hypothesis testing, the FDR (false discovery rate) was calculated using the qvalue package for R. All the genes with a FDR less than 0.01 were used in subsequent analyses. All the calculations were performed using the R statistical software (http://www.r-project.org).

Evaluation of miRNAs expression by quantitative RT-PCR

For each sample and for each miRNA, 5 ng of total RNA was converted to cDNA by TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) with the miRNA specific primer contained in the TaqMan MicroRNA assays (Applied Biosystems). MiRNA expression was evaluated in 10 µl total volume by quantitative RT-PCR following the manufacturer protocol in the presence of 1× TaqMan Universal Master Mix (Applied Biosystems) on a 7900HT SDS instrument (Applied Biosystems). Expression differences of miRNAs among samples were determined by the comparative method according to User Bulletin #2 (Applied Biosystems) using the noncoding RNA RNU44 and RNU6B as reference gene.

MiRNA in situ hybridization analysis

Locked nucleic acid (LNA) probes with complementarity to miR-9*, miR-29b, and miR-150 were labelled with 5′-biotin and synthesised using Exiqon (Vedbaek, Denmark). Tissue sections were digested with ISH protease 1 (Ventana Medical Systems, Milan, Italy) and ISH was performed as we previously described (PMID:27618837). Positive (U6; Exiqon) and negative scrambled LNA probes (Exiqon) were used as controls. Only cytoplasmic miRNA staining was retained for scoring purposes.

Network analysis

Experimentally validated target genes (only target genes marked as “strong evidence”) of differentially expressed miRNAs were retrieved from MirTarBase 4.5 database [52]. Among the predicted targets, only those included in the GO category “lymphocyte differentiation” (GO:0030098) were kept. Using the filtered targets and a database of protein interactions (http://dp.univr.it/~laudanna/LCTST/downloads/index.html), comprising 14642 proteins and 270062 edges, we generated subnetworks which included the shortest paths joining the primary targets. Shortest paths were calculated with PeSea application 3.0 version implemented in Cytoscape 3.0 [53].
Gene ontology and pathway analysis

A comprehensive list of first interactors of genes which are nodes of the shortest paths was submitted to Panther (Protein ANalysis THrough Evolutionary Relationships) Classification System at http://pantherdb.org/) for evaluation of enrichment of annotations for functional classification and cellular pathways [54, 55]. For gene ontology and pathway analysis, a significant value threshold of 0.05 was applied.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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