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Immunodeficiency, centromeric region instability, cacial anomalies (ICF; OMIM #242860) syndrome, due to mutations in the DNMT3B gene, is characterized by inheritance of aberrant patterns of DNA methylation and heterochromatin defects. Patients show variable agammaglobulinemia and a reduced number of T cells, making them prone to infections and death before adulthood. Other variable symptoms include facial dysmorphism, growth and mental retardation. Despite the recent advances in identifying the dysregulated genes, the molecular mechanisms, which underlie the altered gene expression causing ICF phenotype complexity, are not well understood. Held the recently-shown tight correlation between epigenetics and microRNAs (miRNAs), we searched for miRNAs regulated by DNMT3B activity, comparing cell lines from ICF patients with those from healthy individuals. We observe that 89 miRNAs, some of which involved in immune function, development and neurogenesis, are dysregulated in ICF (LCLs) compared to wild-type cells. Significant DNA hypomethylation of miRNA CpG islands was not observed in cases of miRNA upregulation in ICF cells, suggesting a more subtle effect of DNMT3B deficiency on their regulation; however, a modification of histone marks, especially H3K27 and H3K4 trimethylation, and H4 acetylation, was observed concomitantly with changes in microRNA expression. Functional correlation between miRNA and mRNA expression of their targets allow us to suppose a regulation either at mRNA level or at protein level. These results provide a better understanding of how DNA methylation and histone code interact to regulate the class of microRNA genes and enable us to predict molecular events possibly contributing to ICF condition.

Introduction

ICF syndrome is the only human pathology exhibiting Mendelian inheritance of aberrant DNA methylation due to mutations in one of the DNA methyltransferase genes (the de novo methyltransferase DNMT3B). Sixty-seventy percent of analyzed patients had mutations in both alleles of Dnmt3B within the coding portion.[1-3] These are often missense mutations[4-7] and they are frequently found in C-terminal portion of the protein containing the catalytic domain. These findings suggest the loss of DNA methyltransferase activity, and not some other functions of DNMT3B protein, to be responsible for the disease. In addition, since deletions of the Dnmt3b catalytic domain in mice results in prenatal lethality,[6] mutations in DNMT3B are likely to leave residual activity in ICF patients,[8] which is consistent with the phenotype of mice homozygous for ICF mutations as recently described.[9]

DNA hypomethylation in ICF seems to be targeted to specific sequences, such as satellite DNA 2 and 3 of the juxtacentromeric heterochromatin at chromosome 1, 16 and sometimes 9, which show aberrant rearrangements in mitogen-stimulated lymphocytes, even in interphase.[10] Although the hypomethylation of classical satellite 2 and 3 DNA is extended to other tissues and cell cultures from ICF patients, such as fibroblasts, cytological abnormalities are almost always limited to lymphocytes.

At present, it is still unclear how mutations in DNMT3B and the resulting deficiency in DNA methyltransferase activity cause ICF syndrome. In particular, it is not apparent why the impaired DNMT3B activity results mainly in immunodeficiency. Even though several lines of evidence suggest that DNA methylation is critical for immune cell lineages, the specific gene targets of DNA methylation and the molecular pathways underlying the specific phenotypes observed in these cells, such as chromosomal instability and agammaglobulinemia, are still obscure. The expression of several hundred genes is aberrant in ICF cells, being both up and downregulated. Bioinformatics analysis shows that they are critical for immune function, development and neurogenesis, which are highly relevant to the ICF phenotype.[11,12]

Subtle but significant reduction of promoter methylation was seen only in few genes having higher RNA levels in ICF than in control LCLs.[11,13] This raises the question about the relationship between hypomethylation of specific repetitive DNA sequences in ICF patients and altered expression of genes not regulated by promoter methylation.

MicroRNAs (miRNAs) are a class of small, endogenous noncoding RNA molecules that posttranscriptionally regulate gene expression. The function of only a small fraction of these has been described in detail and point to their involvement in a variety of developmental and physiological processes.[14,15]

Stimulating advances in genome function are suggesting a close connection between epigenetics and microRNAs (miRNAs). There are emerging views in two directions. One is about the effect of microRNAs on epigenetic machinery and the other is about the control of miRNA expression by epigenetic mechanisms.[16] Thus, a mechanism that would explain the deregulation of genes not showing promoter hypomethylation observed in ICF cells could involve an altered epigenetic regulation of microRNAs with essential roles in ICF pathogenesis.

In the present study, we had two main goals: (1) to better understand whether dysregulation of microRNA function may contribute to the phenotypic effects characteristic of ICF syndrome patients and (2) to use ICF syndrome as a model system to understand at what extent DNMT3B activity is targeted to microRNA promoter regions and the interplay between DNMT3B function and the other epigenetic modifications in their regulation.

In order to address these goals, we performed genome-wide microRNA expression profiling in lymphoblastoid cells and fibroblasts derived from ICF patients compared to normal cell lines. We demonstrate distinct changes in the expression of some miRNAs, when comparing ICF cell lines versus normal ones. A tissue-specific comparison between lymphoblastoid cells and fibroblasts from ICF patients yielded a list of different up and downregulated microRNAs that could account for some ICF phenotypic aspects.

Loss of DNA methylation was not found at upregulated microRNA CpG islands that we analyzed comparing ICF and normal LCLs. Thus, deficiency in methyltransferase activity of DNMT3B in ICF cells does not seem to impair the methylation status of CpG islands associated to dysregulated microRNAs. Nonetheless, chromatin immunoprecipitation experiments showed that histone modification patterns at genomic region surrounding affected microRNAs were clearly altered. The acquisition of histone H3 lysine 4 and lysine 27 trimethylation, and histone H4 acetylation were the most consistent alterations in ICF relative to normal LCLs.

Enrichment or depletion of specific miRNAs in ICF cells is correlated with corresponding reduction or increase of expression of mRNA transcripts harboring seed matches to these micro-RNAs as being predicted or validated target genes. Most of these affected target genes were classified as involved in molecular pathways impaired in ICF syndrome,[11] namely immune function, development and neurogenesis. Thus, it

suggests that their microRNA-mediated regulation can be an important contributing factor to the ICF phenotype genesis.

In sum, our analysis shows that loss of DNMT3B function leads to the alteration of an additional level of gene expression regulation in ICF cells, provided by the microRNA molecules. These findings pave the way to further understand the molecular pathogenesis of ICF syndrome and, more intriguingly, to unravel the intricate web of epigenetic interactions.
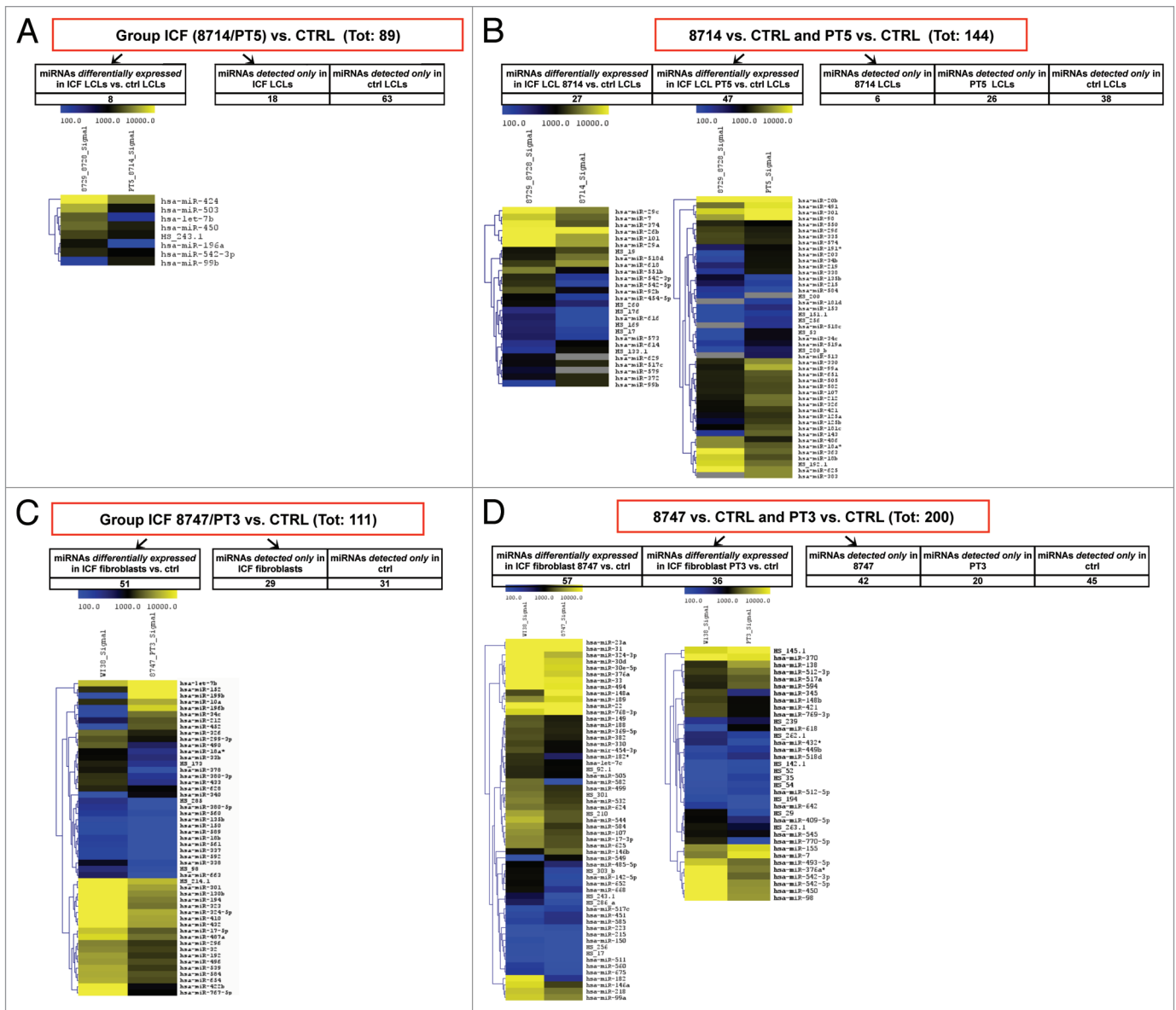
Results

Global expression profiles define microRNA differentially expressed in ICF versus normal LCLs and fibroblasts. How the limited amount of DNA demethylation, due to the impaired DNMT3B activity, can cause the immunodeficiency and other variable symptoms associated with ICF syndrome remains still obscure. Since microRNAs can contribute to the regulation of numerous biological processes by inactivating hundreds of target genes, we hypothesize that epigenetic deregulation of these molecules could account for altered gene expression in ICF cells compared to normal cells.

To this aim, we analyzed global microRNA expression patterns using Illumina's microRNA expression profiling panels in two LCLs derived from normal individuals (GM08728 and GM08729) and two LCLs derived from ICF patients (PT5 and GM08714). MicroRNA expression profiles in fibroblast cell lines from control (WI38) and ICF patients (PT3 and GM08747) were also compared to get a broader picture of DNMT3B deficiency in microRNA regulation and to open new points of view for the study of their contribution to ICF phenotype.

ICF patients show different mutations in Dnmt3B gene: PT5 and P4 (GM08714/47) are compound heterozygous individuals (V699G/R54X and A603T/STP807ins respectively), PT3 is homozygous (STP807ins).[4]

Human microRNA expression profiling panels from Illumina measure the levels of 735 miRNAs, of which 470 are described in the miRBase database v9.1,[17] and 265 are identified in a RAKE analysis study.[18,19] Data analyses were performed with Illumina BeadStudio v3.3 software and normalized with the average normalization algorithm: microRNAs were considered as detected if the detection p-value was lower than 0.05. To identify differentially-expressed microRNAs a Differential Score ≥|20|, which is a qualitative measure to assess an effective variation was considered (see methods).

Overall, the analysis based on grouping ICF LCLs yielded 89 dysregulated microRNAs: 8 microRNAs differentially expressed in ICF samples, PT5 and GM08714 relative to normal cells (1 upregulated and 7 downregulated; **Fig. 1A**, left), whereas 18 microRNAs were expressed only in ICF samples and 63 only in control samples (**Fig. 1A**, right). Hierarchical clustering highlights the distinctive microRNA expression profile of normal and ICF samples, even though specific differences appear when we compare ICF cell lines one another (data not shown). Since ICF cells derive from patients having different Dnmt3B

Figure 1. Schematic representation of microarray results for LCLs and fibroblasts, divided for group analysis and single sample analysis. (A) Dysregulated microRNAs in the group of ICF (PT5 and 8714) and control (8728 and 8729) lymphoblastoid cells lines (LCLs). Left, reports microRNAs significantly differentially-expressed (Diff Score ≥|20|) in ICF LCLs vs. controls and the corresponding heat map derived from the experiment. Right, shows the number of microRNAs exclusively expressed in ICF or control groups. (B) Dysregulated microRNAs in single ICF LCLs (PT5 or 8714) compared with the group of controls. Left and right, show microRNAs significantly differentially-expressed and microRNAs detected only in patients or controls respectively. (C and D) Same as above by grouping ICF fibroblast samples (8747 and PT3) compared to control fibroblast (WI38) (C), or by analyzing single ICF fibroblasts versus control (D).

mutations leading to variable ICF phenotypes, we also reported results from each ICF cell line separately compared to the group of control cells. In this case we found 144 dysregulayted miRNAs: 27 differentially expressed miRNAs in 8714 and 47 in PT5 compared to control samples (**Fig. 1B**, left). In addition, 6 microRNAs were detected only in 8714, 26 only in PT5 and 38 only in controls (**Fig. 1B**, right).

The analysis of both ICF fibroblasts versus control cells resulted in 111 dysregulated miRNAs: 51 differentially expressed miRNAs (9 upregulated and 42 downregulated; **Fig. 1C**, left); 29 microRNAs found only in ICF samples and 31 only in control

sample (**Fig. 1C**, right). However, again some heterogeneity in microRNA expression was observed between the two ICF cell lines since several microRNAs were found differentially expressed or detected in the single ICF sample versus control (**Fig. 1D**, left and right respectively).

To select microRNAs with the largest differential expression, ratio ≥|1.5| of each signal in ICF cells against the average reference signal in control cells was considered. The ratio gives a quantitative measure of the difference of expression between the samples. **Table 1** reports upregulated and downregulated microRNAs in ICF LCLs and fibroblasts vs. control cells

Table 1. Upregulated and downregulated miRNAs in ICF LCLs and fibroblasts vs. control samples

hsa-miRNA-gene	ICF vs. control	Chr.	miRNA cluster	CpG plot	CpG searcher	Chromosomal location
hsa-miR-153	↑	2		1	1	Overlapping transcript
hsa-miR-203	↑	14		1	1	Intergenic
hsa-miR-212	↑	17		5	3	Intergenic
hsa-miR-34b	↑	11	1	4	2	Intergenic
hsa-miR-34c	↑	11	1	4	2	Intergenic
hsa-miR-369	↑	14		4	3	Intergenic
hsa-miR-518c	↑	19		no	1	Intergenic
hsa-miR-219	↑	6		2	2	Intergenic
hsa-miR-219	↑	9		3	2	Intergenic
hsa-miR-522	↑	19	2	no	3	Intergenic
hsa-miR-618	↑	12		2	1	Overlapping transcript
hsa-miR-138	↑	3		no	no	Intergenic
hsa-miR-143	↑	5	3	no	no	Intergenic
hsa-miR-383	↑	8		no	no	Overlapping transcript
hsa-miR-517c	↑	19	2	no	no	Intergenic
hsa-miR-518d	↑	19	2	no	no	Intergenic
hsa-miR-18b	↓	X	4	6	2	Intergenic
hsa-miR-296	↓	20		1	1	Intergenic
hsa-miR-215	↓	1		2	1	Overlapping transcript
hsa-miR-542	↓	X	5	2	1	Intergenic
hsa-miR-149	↓	2		1	1	Overlapping transcript
hsa-miR-18a*	↓	13		1	1	Overlapping transcript
hsa-miR-410	↓	14		4	3	Intergenic
hsa-miR-432	↓	14	6	1	3	Intergenic
hsa-miR-95	↓	4		no	1	Overlapping transcript
hsa-miR-494	↓	14	7	no	2	Intergenic
hsa-miR-487a	↓	14	8	no	1	Intergenic
hsa-miR-486	↓	8		no	1	Overlapping transcript
hsa-mir-376a-1	↓	14	9	2	2	Intergenic
hsa-mir-376a-2	↓	14	9	2	2	Intergenic
hsa-miR-323	↓	14	7	no	2	Intergenic
hsa-miR-329	↓	14	7	no	2	Intergenic
hsa-miR-487b	↓	14	8	no	no	Intergenic
hsa-miR-625	↓	14		no	no	Overlapping transcript
hsa-miR-135b	↓	1		no	no	Overlapping transcript
hsa-miR-29c	↓	1		no	no	Intergenic
hsa-miR-450	↓	X	5	no	no	Intergenic
hsa-miR-584	↓	5		no	no	Overlapping transcript
hsa-miR-511	↓	10		no	no	Overlapping transcript

Upregulated and downregulated miRNAs common to ICF LCLs and fibroblasts including those differentially expressed (DiffScore ≥|20|) showing the greatest difference (ratio ≥|1.5|) between ICF and controls, and miRNAs exclusively detected in samples or controls. Chromosome position, cluster affiliation, CpG island association (analyzed by CpG plot and CpG searcher) and relative position to overlapping transcripts are reported. Chr, Chromosome. Grey shading remarks miRNAs associated to one or more than one CpG island within 4 kilobases surrounding miRNAs.

according to this parameter, and includes miRNAs exclusively detected in ICF or controls. The class of microRNAs named HS_contained in the Illumina microarray was excluded from this list and from following experiments of the present study because they are generally less characterized and not included in the miRbase Sanger database.

Common affected microRNAs in fibroblasts and LCLs ICF have been identified representing about 20% of total

micro-RNAs dysregulated in LCLs alone. Some of these microRNAs are probably involved in housekeeping pathways altered in ICF syndrome. MicroRNAs affected only in ICF LCLs could account for tissue specific functions and/or anomalies, such as chromosomal aberrations at pericentromeric heterochromatin of chr. 1, 16 and 9 observed in lymphocytes but not in fibroblasts of ICF patients[10] (**Table 2A and B**). Most miRNAs that are organized in clusters in close proximity to a chromosome have similar expression levels, indicating the possibility of being transcribed in polycistronic fashion under the same promoter. From our data, miR-34b and miR-34c located in a cluster on chromosome 11 exhibit the same expression profile in ICF cells. Likewise, miR-99b, miR-125a and let7e, which are co-located in a cluster on chromosome 19, are all upregulated in ICF LCLs compared to control cells. Four microRNAs, miR-424, miR-450, miR-542-3p and miR-542-5p, clustered on X chromosome are significantly downregulated in ICF samples, suggesting again the sharing of regulatory features. Overall, we found 18 clusters of microRNAs sharing the expression profile in ICF cells.

We analyzed the chromosomal location of dysregulated microRNAs and indicated when their mature sequences were intergenic or included in overlapping transcripts. The correlation between microRNAs and host genes expression profiles was also examined (see below).

In order to highlight the presence of predicted CpG islands in microRNA surrounding genomic region, we used CpG Island Searcher (http://cpgislands.usc.edu/) and CpG Plot (www.ebi.ac.uk/Tools/emboss/cpgplot/index.html), analyzing up to 2 kb upstream and 2 kb downstream the upregulated and downregulated microRNAs. For some microRNAs, e.g., miR199a/a* and miR-34b/c, the association with a functional CpG island was already known and also functionally characterized.[20,21]

We found that roughly half of dysregulated microRNAs reported in **Tables 1, 2A and B** are associated to one or more regions showing features of CpG island (grey shading). This suggests a DNA methylation mediated mechanism of transcriptional regulation for these microRNAs.

Among microRNAs whose expression profile was altered in ICF samples compared to controls, we identified a number of them known to play an important role in the biological processes, which prevalently are impaired in the disease. This aspect will be discussed below.

Validation of the miRNA expression microarray data provides a list of potential DNMT3B-regulated microRNAs. The microarray analysis described in the previous section yielded a large catalogue of microRNAs that may be regulated directly or indirectly by DNA methylation and DNMT3B transcriptional repression activity.

We then selected 15 microRNAs for follow-up confirmation using quantitative Real Time PCR technique in LCLs and fibroblasts samples. The choice criteria for microarray results validation and further examinations included: (i) both upregulated and downregulated microRNAs showing the largest ratio, (ii) microRNAs associated with CpG islands in the surrounding genomic region (putative and/or experimentally characterized) and (iii) microRNAs already known to be somehow associated

to immune function and nervous system. The majority of them demonstrated the expected differences in expression between normal and ICF cells predicted by the microarray analysis (**Fig. 2A and B**).

This expression analysis confirms similarities and differences among ICF syndrome lines and suggests that the heterogeneity could be due to distinct Dnmt3B mutations reported. To validate the microarray results, TaqMan based qRT-PCR was carried out using the relative quantification method. Results were normalized with endogenous control RNU44 and the fold change was calculated by equation $2^{-\Delta\Delta Ct}$ (see methods).

Functional correlation between miRNAs and predicted and/or validated mRNA target in ICF syndrome. It is known that miRNAs can downregulate mRNA expression by controlling mRNA stability or cleaving target mRNAs. Thus, we analyzed the functional correlation between miRNAs and mRNA expression in ICF cells to identify differentially expressed miRNA-mRNA modules and to assess the extent of miRNA effects on mRNA expression. While directly comparing the known lists of dysregulated miRNAs and mRNAs in ICF samples, we also searched for predicted and/or validated miRNA-mRNA pairs potentially contributing to the ICF phenotype.

Therefore, we exploited the previously reported results of gene expression profiling performed on the same ICF LCLs used in this study.[11] To get an overall picture of functional correlation between dysregulated mRNA and target of dysregulated microRNA, we queried the MMIA (microRNA and mRNA integrated analysis) software, an integrated web tool for a combined examination of microRNA and mRNA expression.[22] Complete data sets from both microRNAs and mRNA microarrays performed on ICF samples and controls were compared, and the intersection of TargetScan 5.2 and PITA (probability of interaction by target accessibility) was preferably used to obtain predicted targets. A significant inverse functional correlation was detected comparing both upregulated mRNAs with targets of downregulated miRNAs and downregulated mRNAs with targets of upregulated miRNAs (p value 0.04 and 0.01, respectively by using Fisher's exact test), thus indicating that involved microRNAs may preferentially mediate mRNA degradation rather than translational repression.

Among the dysregulated microRNAs resulting from microarray experiment, we focused on specific microRNAs validated by Real Time PCR to analyze their potential relevance to ICF phenotype. Consequently, we specifically compared: (i) downregulated mRNAs and targets of 11 chosen upregulated miRNAs and (ii) upregulated mRNAs and targets of two downregulated miRNAs for pairs of ICF and control samples. This analysis enabled us to identify several predicted and/or validated mRNA targets of dysregulated microRNAs, whose transcript level were oppositely changed. **Table 3** shows the results from the inverse correlation analysis of selected microRNAs differentially expressed in ICF and their targets. A meta miRNA predictor (MAMI; http://mami.med.harvard.edu/) enabling maximal accuracy and tuneable sensitivity and specificity in predictions was applied to predict targets of differentially expressed miRNAs.

Table 2A. Upregulated miRNAs in only ICF LCLs vs. control samples

hsa-miRNA-gene	ICF vs. control	Chr.	miRNA cluster	CpG plot	CpG searcher	Chromosomal location
hsa-let-7e	↑	19	10	2	2	Intergenic
hsa-miR-125a	↑	19	10	2	2	Intergenic
hsa-miR-99b	↑	19	10	2	2	Intergenic
hsa-miR-125b	↑	11		1	1	Intergenic
hsa-miR-125b	↑	21		no	1	Overlapping transcript
hsa-miR-181c	↑	19	11	1	2	Intergenic
hsa-miR-181d	↑	19	11	1	2	Intergenic
hsa-miR-191*	↑	3		5	4	Intergenic
hsa-miR-199a	↑	19		no	1	Overlapping transcript
hsa-miR-220b	↑	19		5	4	Intergenic
hsa-miR-220c	↑	19		no	1	Intergenic
hsa-miR-301	↑	22		2	2	Overlapping transcript
hsa-miR-301	↑	17		3	2	Overlapping transcript
hsa-miR-338	↑	17		no	2	Overlapping transcript
hsa-miR-372	↑	19		1	2	Intergenic
hsa-miR-505	↑	X		no	1	Overlapping transcript
hsa-miR-519a-1	↑	19	12	no	1	Intergenic
hsa-miR-519a-2	↑	19	12	no	1	Intergenic
hsa-miR-559	↑	2		5	3	Overlapping transcript
hsa-miR-593	↑	7		1	1	Overlapping transcript
hsa-miR-636	↑	17		2	1	Overlapping transcript
hsa-miR-107	↑	10		no	no	Overlapping transcript
hsa-miR-145	↑	5	3	no	no	Intergenic
hsa-miR-181a	↑	1	13	no	no	Overlapping transcript
hsa-miR-181a	↑	9	14	no	no	Overlapping transcript
hsa-miR-181b	↑	1	13	no	no	Overlapping transcript
hsa-miR-181b	↑	9	14	no	no	Overlapping transcript
hsa-miR-199a	↑	1		no	no	Overlapping transcript
hsa-miR-220a	↑	X		no	no	Intergenic
hsa-miR-326	↑	11		no	no	Overlapping transcript
hsa-miR-421	↑	X		no	no	Intergenic
hsa-miR-506	↑	X	15	no	no	Intergenic
hsa-miR-513b	↑	X		no	no	Intergenic
hsa-miR-582	↑	5		no	no	Overlapping transcript
hsa-miR-614	↑	12		no	no	Intergenic
hsa-miR-617	↑	12		no	no	Overlapping transcript
hsa-miR-651	↑	X		no	no	Intergenic
hsa-miR-98	↑	X		no	no	Overlapping transcript
hsa-miR-99a	↑	21		no	no	Overlapping transcript

Upregulated and downregulated miRNAs only in ICF LCLs compared to controls. The criteria of classification are the same as for Table 1.

Target genes mainly related with the disease class according to Gene Ontology as described in Jin et al. are indicated in italics and underlined. Most of the interesting examples include miR181a, mir-181b, miR-125b and miR-34c, all upregulated and known to have among mRNA targets possibly related to specific ICF immune phenotype, that is an immunodeficiency due to defective B terminal differentiation and consequent absence of memory and plasma cells, contributing to agammaglobulinemia.[23]

TNFSF11 [tumor necrosis factor (ligand) superfamily, member 11], which is a predicted target of miR181a, is a cytokine involved in the regulation of T cell-dependent immune response[24] and the deficient mice exhibited defects of T and B lymphocytes differentiation.[25] Even more interestingly, TCL1A

Table 2B. Downregulated miRNAs only in ICF LCLs vs. control samples

| hsa-miRNA-gene	ICF vs. control	Chr.	miRNA cluster	CpG plot	CpG searcher	Chromosomal location
hsa-miR-101	↓	9		1	1	Overlapping transcript
hsa-miR-127	↓	14	6	2	2	Intergenic
hsa-miR-134	↓	14	8	1	no	Intergenic
hsa-miR-136	↓	14	6	2	2	Intergenic
hsa-miR-154*	↓	14	8	1	no	Intergenic
hsa-miR-196a	↓	17		3	2	Intergenic
hsa-miR-196a	↓	12		no	1	Intergenic
hsa-miR-202*	↓	10		4	4	Overlapping transcript
hsa-miR-20b	↓	X	4	3	2	Intergenic
hsa-miR-26b	↓	2		4	1	Overlapping transcript
hsa-miR-329-1	↓	14	7	4	4	Intergenic
hsa-miR-329-2	↓	14	7	4	4	Intergenic
hsa-miR-335	↓	7		2	2	Overlapping transcript
hsa-miR-340	↓	5		2	2	Overlapping transcript
hsa-miR-363	↓	X	4	3	2	Intergenic
hsa-miR-374a	↓	X		no	1	Overlapping transcript
hsa-mir-376a-1	↓	14	16	2	2	Intergenic
hsa-mir-376a-2	↓	14	16	2	2	Intergenic
hsa-miR-411	↓	4	7	4	4	Intergenic
hsa-miR-424	↓	X	5	3	4	Overlapping transcript
hsa-miR-452	↓	X		1	2	Overlapping transcript
hsa-miR-483	↓	11		8	5	Overlapping transcript
hsa-miR-515-1	↓	19	17	1	2	Intergenic
hsa-miR-515-2	↓	19	17	1	2	Intergenic
hsa-miR-520a*	↓	19	17	1	2	Intergenic
hsa-miR-550	↓	7		1	1	Overlapping transcript
hsa-miR-566	↓	3		1	2	Overlapping transcript
hsa-miR-579	↓	5		no	1	Overlapping transcript
hsa-miR-590	↓	7		2	2	Overlapping transcript
hsa-miR-607	↓	10		3	2	Intergenic
hsa-miR-616	↓	12		3	4	Overlapping transcript
hsa-miR-661	↓	8		3	3	Overlapping transcript
hsa-miR-7-1	↓	9		1	2	Overlapping transcript
hsa-miR-7-2	↓	15		no	1	Intergenic
hsa-miR-92b	↓	1		4	2	Intergenic
hsa-let-7b	↓	22		no	no	Overlapping transcript
hsa-miR-101	↓	1		no	no	Intergenic
hsa-miR-187	↓	8		no	no	Intergenic
hsa-miR-29a	↓	7		no	no	Overlapping transcript
hsa-miR-302a*	↓	4	18	no	no	Overlapping transcript
hsa-miR-302b	↓	4	18	no	no	Overlapping transcript
hsa-miR-302c*	↓	4	18	no	no	Overlapping transcript
hsa-miR-454	↓	17		no	no	Overlapping transcript
hsa-miR-455	↓	9		no	no	Overlapping transcript
hsa-miR-508	↓	X	15	no	no	Intergenic
hsa-miR-511-1	↓	10		no	no	Overlapping transcript

Upregulated and downregulated miRNAs only in ICF LCLs compared to controls. The criteria of classification are the same as for Table 1.

Table 2B. Downregulated miRNAs only in ICF LCLs vs. control samples

hsa-miRNA genes	ICF vs. control	Chr.	miRNA cluster	CpG Plot	CpG Searcher	Chromosomal location
hsa-miR-511-2	↓	10		no	no	Overlapping transcript
hsa-miR-520b	↓	19		no	no	Intergenic
hsa-miR-551b	↓	3		no	no	Overlapping transcript
hsa-miR-573	↓	4		no	no	Intergenic
hsa-miR-629	↓	15		no	no	Overlapping transcript
hsa-miR-7-3	↓	19		no	no	Overlapping transcript

Upregulated and downregulated miRNAs only in ICF LCLs compared to controls. The criteria of classification are the same as for Table 1.
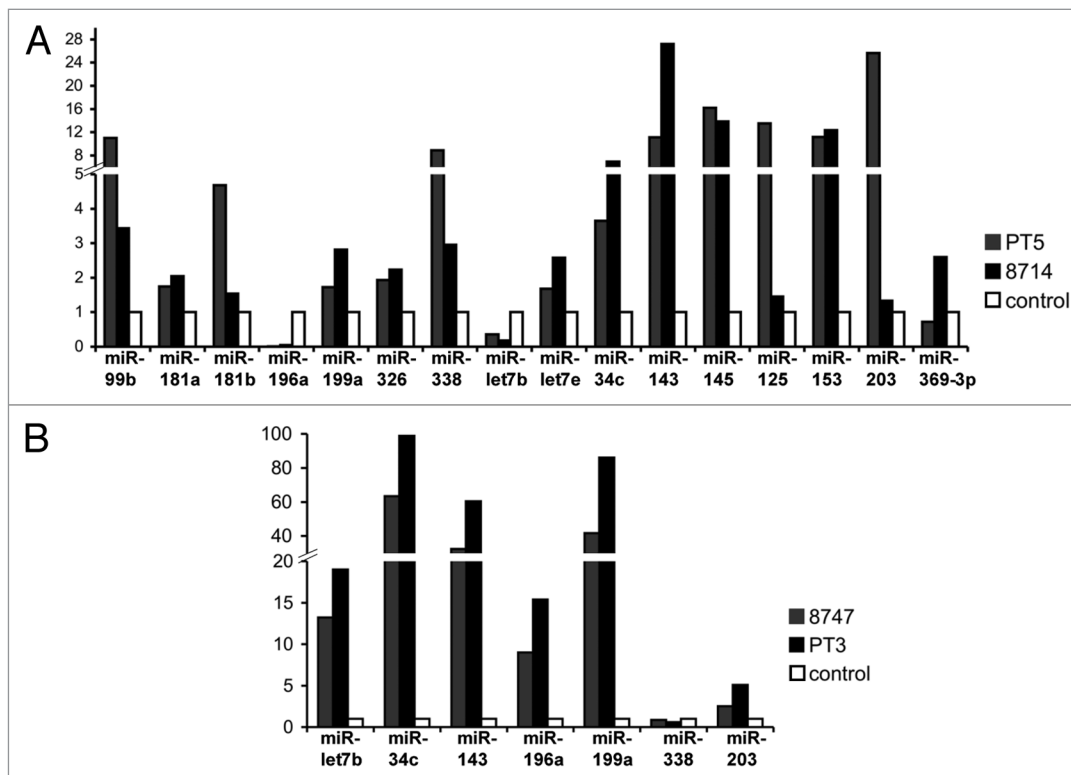
Figure 2. TaqMan based qRT-PCR analysis of a subset of up and downregulated miRNAs in ICF LCLs, PT5 and 8714, (A) and in fibroblasts, 8747 and PT3, (B) compared to normal cells resulting from microarray experiments. The results were normalized with endogenous control RNU44 and the fold change was calculated by using the equation $2^{-\Delta\Delta Ct}$.

(T-cell leukemia/lymphoma 1A) appears to be critical for a proper immune function since its deficiency compromises lymphopoiesis at pre-B stage impairing IgG1 and IgG2b production.[26] TCL1A has been shown to be a validated gene target of miR-181b.[27]

Beside inversely dysregulated miRNA-mRNA modules resulting from microarray experiments, several putative miRNA-mRNA pairs could be predicted to contribute to the ICF condition. Since it has been supposed that the expression of candidate genes for interfering with later stages of B cell differentiation and maturation should be impaired in ICF cells, the validated mRNA target of miR-125b PRDM1,[28] as well as the predicted mRNA target of miR-34c, XBP-1, appeared to be interesting examples. Expression of PRDM1 (PR domain containing 1, with ZNF domain), in B cells committed to the plasma cell lineage is required for the activation of XBP-1 and for the development of antibody-secreting plasma cells. The

X-box binding protein 1 (XBP-1), essential for differentiation of plasma cells, is a transcription factor containing a bZIP domain. It controls the expression of IL-6, which promotes plasma cells growth and immunoglobulins production in B lymphocytes.[29]

Together with miR-125 family, the miR-199 family as well as miR-138 show a distinct expression in GC B cells although none of these two miRNAs has been investigated for a role in this cell compartment yet.[30]

In addition to the immune function, several mRNA targets of identified miRNAs, involved in the other ICF variably impaired biological processes, such as neuronal and brain function, neurogenesis and development, were found inversely dysregulated in these disease cells (**Table 3**). MiR-196 family is known to be transcribed from intergenic regions within Hox genes clusters in vertebrates and includes mir-196a1, mir-196a2 e mir-196b. As for the Hox genes, these microRNAs seem to control

**Table 3.** Inversely dysregulated microRNAs and mRNAs relevant to ICF phenotype

Upregulated microRNAs in ICF syndrome	Downregulated mRNA targets in ICF samples predicted (MAMI) and/or validated	Enriched biological processes of dysregulated miRNA targets using PANTHER
hsa-mir-181a	*ADM*, ADRBK1, BCL2L11, *BNC2*, FKSG44, GPR157, *GRM7*, *KIAA1212*, LARP6, LOC96610, MAP1B, *MGMT*, *RUNX1*, TEAD1, *TNFSF11*, VRK3, *CD69*	neurogenesis, development, neuronal and brain function, immune and cellular defence
hsa-mir-181b	*ADM*, BCL2L11, *BNC2*, FAM13A1, FKSG44, *KIAA1212*, LARP6, *LOC96610*, MAP1B, *MGMT*, *NFAT5*, PDZRN3, *RUNX1*, TEAD1, *TNFSF11*, VRK3, *TCL1A*	neurogenesis, development, immune and cellular defence
hsa-mir-125b	C1orf93, *CACNB1*, KCNIP3, *MAP2K7*, *PLK3*, RALGPS2, TMEM77, *VDR*, *SASH1*, *FLII*, SLC2A5, *IRF4*	development, immune and cellular defence
hsa-mir-125a	*CACNB1*, KCNIP3, *MAP2K7*, RALGPS2, TMEM77, UBE2J1, *VDR*	development, neuronal and brain function
hsa-mir-99b	*HLA-DOA*, *FHL1*, FKSG44, *SPG20*, PAG1, POLN, RPS27A, SPDEF, SPPL2B, *SSR3*	neurogenesis, development, immune and cellular defence
hsa-mir-34c	*ALOX5*, ARHGAP26, BIK, *BNC2*, *CACNB1*, DDX17, FUT8, GJB3, JAKMIP1, PEX16, *PRKACB*, RALGPS2, TOX, WASF1	neurogenesis, development, neuronal and brain function, immune and cellular defence
hsa-mir-34b	ARHGAP26, *BNC2*, *CACNB1*, DDX17, FUT8, GJB3, JAKMIP1, *PRKACB*, RALGPS2, SLC5A8, WASF1, *NFAT5*, *ALOX5*, *ADAM23*, *ID3*, *BCL11A*, *COL5A1*, *FHL1*	neurogenesis, development, neuronal and brain function, immune and cellular defence
hsa-mir-338	DNAH17, TMEM2, IKZF3, *ID3*, KLF10, ELL2, PDE4DIP, ESCO1, MAPKAPK5, ABCG1, SOX2, *POU2F2*, LARP6, ADRBK1, *CNOT6L*, MARK3, MAP1B, *KIAA1212*, *ISL2*, *LCK*, SNCA, TERF2, SPTBN4, SLC2A3	neurogenesis, development
hsa-mir-199a	*CD24*, *LCK*, TMEM2, PDE4DIP, PDZRN3, *COL5A1*, ABCG1, DIAPH1, *POU2F2*, LARP6, LCOR, MARK3, FASTK, FUT8, ZBTB20, MAP1B, MXI1, TERF2, SPTBN4, KCNN3, RALGPS2, SGK	neurogenesis, development, immune and cellular defence
hsa-mir-143	*NFAT5*, KLF10, *FHL1*, *COL5A1*, *CBX8*, MAPK3, DIAPH1, *POU2F2*, SLC9A5, MARK3, FASTK, RAD50, *FES*, MXI1, *OAT*, TERF2, *NFATC2*, *ITM2B*	neurogenesis, development, immune and cellular defence
hsa-mir-145	*DNAH17*, NCOA3, *ID3*, ELL2, PDE4DIP, *PRKACB*, *FHL1*, ABCG1, *CACNB1*, DIAPH1, *FAM80B*, PLEKHC1, JAKMIP1, SLC9A5, MARK3, *MYO1D*, FASTK, *HMX2*, MAP1B, *OAT*, SEZ6L2, TERF2, SPTBN4	neurogenesis, development, neuronal and brain function,
Downregulated microRNAs in ICF syndrome	**Upregulated mRNA targets in ICF samples predicted (MAMI) and/or validated**	**Enriched biological processes of identified miRNA targets using PANTHER**
hsa-mir-196a	*LHX2*, FN1, *PTPRG*, *TNFRSF21*, FLNB, KLHL6, SOX5, LMO7, MAT2A, FRY, CASK, DPY19L4, CTNND2, ZNRF1, CSRP2BP, PRPF4B, C10orf38, MYO6, FLRT3, C6orf166, *DLL1*, KLHL13	neurogenesis, development
hsa-let-7b	*ASCL1*, *CCR7*, CPM, DLC1, *FARP1*, FLJ25476, FXYD2, *PTAFR*, SLCO5A1, SOCS1, STARD13, UBE2T, USP6	neurogenesis, development, neuronal and brain function, immune and cellular defence

miRNAs and predicted and/or validated targets (mRNAs) inversely dysregulated in microarray experiments (Illumina and Affymetrix). MAMI software has been exploited to identify transcript targets for the selected microRNAs. Among them, only those inversely dysregulated in microarray and Real Time PCR experiments are listed. Italic and underlined genes are involved in biological processes found enriched in gene expression profiling experiments comparing ICF and control samples by using PANTHER.[9] These correspond to the main impaired functions in ICF patients.

embryonic development of the nervous system.[31] MiR-196a resulted downregulated in ICF cells and, intriguingly, its validated target LHX2,[32] which is crucial for the proper development of cerebral cortex in mouse embryo, shows a marked upregulation in Affymetrix experiments.[11]

To complete the in silico analysis, we also examined those intragenic microRNAs to see whether they were transcribed as part of their hosting transcription unit. In this case, they should share the same expression profile comparing ICF samples with control samples. However, no extensive correlated dysregulation between miRNA and their overlapping transcripts has been found examining all intragenic microRNAs differentially expressed in ICF LCLs.

Impairment of DNMT3B function in ICF syndrome cells does not result in significant changes in DNA methylation patterns at microRNAs examined. To obtain a more detailed analysis of DNA methylation patterns that would allow for an assessment of the contribution of DNA methylation in regulating microRNA expression, we performed bisulfite genomic sequencing [BGS[33]]. We chose four clusters of microRNAs overexpressed in ICF LCLs relative to the normal samples and associated to predicted and/or validated CpG islands: miR-99b/let7e/miR-125a, miR34b/miR-34c, miR-338/miR-657 and miR-199a/miR-199a*. Thus, we designed primers flanking the most CpG-rich regions for each cluster to amplify converted DNA from two ICF LCLs and control cells.
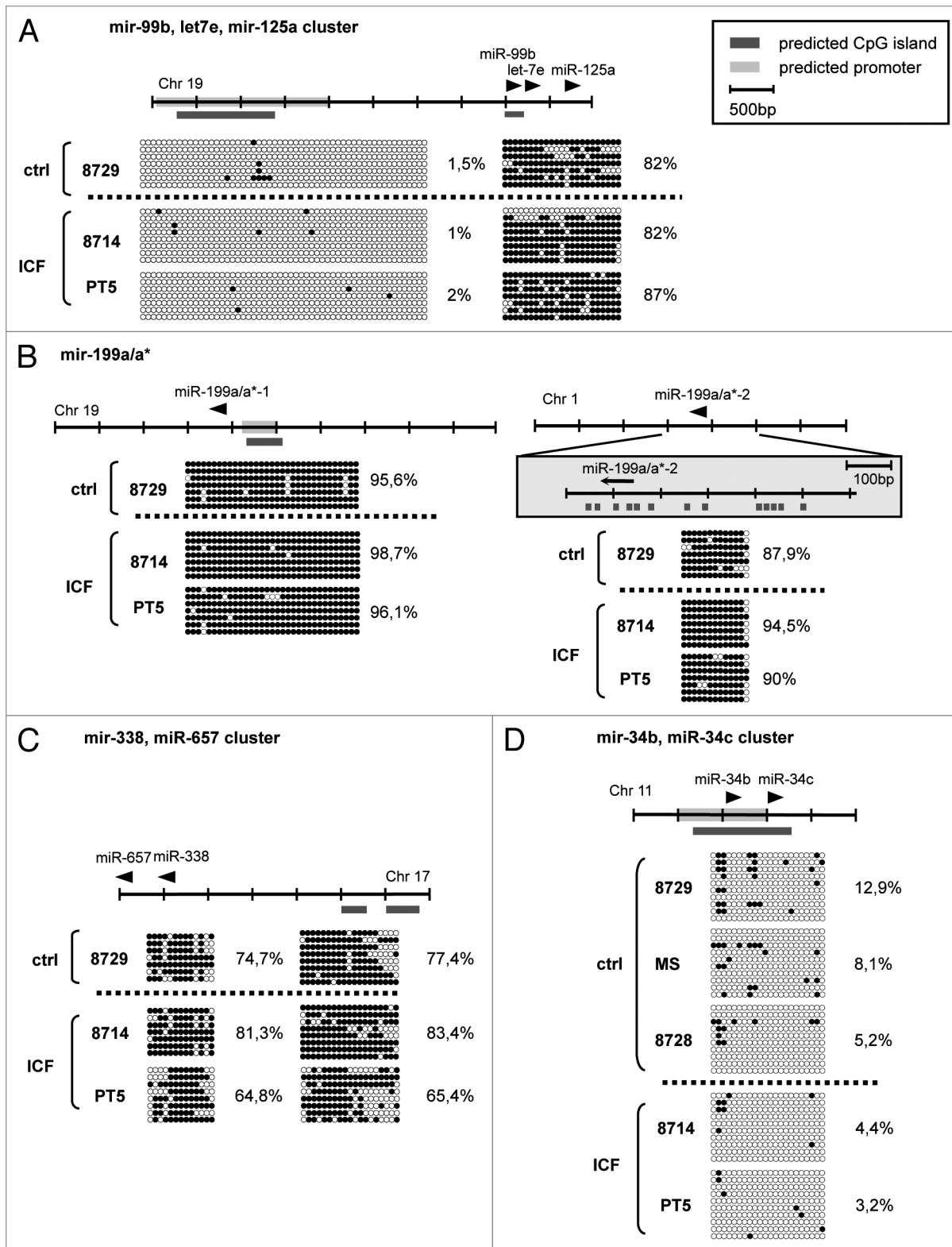
Figure 3. (A–D) Bisulfite genomic sequencing analysis of CpG-rich regions associated to microRNA clusters showing increased expression in ICF syndrome cells. The DNA methylation patterns have been analyzed in two ICF cell lines (8714 and PT5) and one normal cell line (8729). Methylation status of miR-34b/c has been studied in two more control LCLs, 8728 and MS. The predicted promoter, where known, is indicated as grey shading on the bar. Circles below indicate methylation status: filled circles represent methylated CpGs, open circles represent unmethylated CpGs. Each row of circles corresponds to one clone. Following bisulfite treatment and PCR, resulting products were cloned and at least seven independent colonies were sequenced. Percentage of total CpG methylation for all samples is reported.

MiR-99b/let7e/miR-125a cluster is characterized by two CpG-rich regions, one spanning the microRNA mature sequence of miR-99b and let7e and the other about 3 kb upstream (**Fig. 3A**). BGS analysis showed that the first CpG-rich region is highly methylated (about 85% methylation), whereas the second one was almost completely unmethylated (about 1.5%) in normal and ICF cells, suggesting that the latter is probably involved in regulating microRNA transcription. This region has also been described to be associated to predicted transcription start sites based on chromatin modifications identified by ChIP-seq experiments.[34]

However, no difference in DNA methylation level for both CpG islands examined has been found when comparing ICF and control LCLs, indicating that the upregulation of microR-NAs within the cluster on chromosome 19 is not due to loss of methylation. Similar results were obtained when we analyzed the CpG island associated to microRNA cluster including miR-199a/a* on chromosomes 1 and 19 (**Fig. 3B**). BGS analysis revealed high level of DNA methylation in both normal and ICF cells, even though these CpG islands were expected to be hypomethylated in ICF cells since they are described to function as regulative regions of microRNA expression.[20]

As cluster including miR-338 and miR-657 on chromosome 17 also exhibit two CpG-rich regions upstream of the mature microRNA sequences, we compared the DNA methylation profile for both of them in ICF cells and control cells. We could not find any significant difference between 8714 and control: 81.3 and 83.4%, 74.7 and 77.4% total level of DNA methylation at the first and second CpG-rich regions, respectively. However, the ICF cell line PT5 examined by BGS, resulted less densely methylated, showing a 64.8% and 65.4% percent of total DNA methylation level at the two regions (**Fig. 3C**).

We then examined the methylation status of CpG-rich region at miR-34b/c cluster in ICF cells versus three control cell line. This CpG island, also corresponding to the predicted promoter regulating the microRNA transcription, resulted significantly hypomethylated at the second and the third CpGs of the sequence in ICF cells compared to 8729 control cell line (**Fig. 3D**; total CpG methylation 12.5% in control and 4.4 or 3.2% in 8714 and PT5, respectively). χ^2 test (http://faculty.vassar.edu/lowry/VassarStats.html) was used for the statistics (p-value <0.0123). However, when we extended bisulfite analysis to two more control cell lines, MS and 8728, we failed to find the same significant difference as in 8929, even though their total CpG methylation was higher than ICF cells (**Fig. 3D**; total CpG methylation 8.1 and 5.2% respectively). This indicates that differences in DNA methylation profile between patients and controls could be very subtle and variable whenever they occur.

Finally, to look for more informative microRNAs relative to the epigenetic changes leading to ICF phenotype, we chose some microRNAs expressed only in ICF samples to analyze their associated CpG islands by BGS. Methylation profile of miR-203 was studied in LCLs and fibroblasts comparing ICF and control cells, being upregulated in both ICF cell types, whereas BGS of miR-153 and miR-369 was carried out in LCLs, where this microRNA showed exclusive expression in ICF cell lines compared to

control cells. As for previously examined microRNAs, the analysis of these additional miRNAs upregulated in ICF cells did not result in any differential pattern of DNA methylation (**Suppl. Fig. 1A–C**).

All together, BGS data suggest that deficiency of methyl-transferase activity does not appear to result in a significant loss of DNA methylation of CpG-island associated to the microR-NAs analyzed, implying that their upregulation in ICF cells is probably due to indirect effects or to other epigenetic aspects related to DNMT3B function.

Altered microRNA expression in ICF syndrome is associated with changes in the histone code of surrounding genomic regions. Our analysis of DNA methylation profile of four microRNA clusters did not reveal significant DNA methylation differences, even though all analyzed microRNAs were clearly overexpressed in ICF cells with respect to normal cells.

Afterwards, as we wanted to know if aspects of histone code were altered, we performed ChIP analyses using four antibodies, three of which are specific for histone modifications characteristic of transcriptionally active and/or permissive chromatin [histone H3 di or trimethylated on lysine 4, (2- or 3meK4H3), H3 acetylated on lysine 9 (AcK9H3) and histone H4 acetylated, (AcH4)] and one is specific for transcriptionally repressed chromatin [histone H3 trimethylated on lysine 27, (3meK27H3)].[35,36] We focused our analysis on some microRNA clusters examined by bisulfite sequencing, amplifying regions including the mature microRNA sequences and extending it to about 4 kb upstream and 2 kb downstream of the microRNA position. In addition, we also examined two more microRNAs, one upregulated (miR-181a/b) and one downregulated (miR-196) in ICF LCLs, not associated with CpG-rich regions, but apparently significant to normal immune function and skeletal development. In all these cases, we also took into account the prediction of putative promoter regions controlling microRNA expression that has been previously reported in other cell backgrounds.[34]

In general, the histone marks pattern of two or three regions for each microRNA cluster was examined in our ChIP assay. Rabbit IgG was used as a negative control and demonstrated that background binding in ChIP experiments was almost absent.

We first analyzed microRNAs 99b, let7e and 125a, all being overexpressed in ICF cells but not showing differential DNA methylation between ICF and normal samples (**Fig. 4A**). This cluster showed relatively little difference in K9H3 acetylation level, whereas the permissive K4H3 trimethylation and H4 acetylation marks were increased in both ICF samples and specifically enriched only within the two regions (#1 and #2) surrounding the predicted promoter (grey bar). A concomitant reduction of repressive K27H3 trimethylation within the region #2 was also clearly observed. The third region examined (#3), located between the microRNAs mature sequences, showed little difference in histone marks between normal and ICF cells and suggested that this region is probably not involved in the regulation of microRNAs expression. This result, together with the DNA methylation profile of the region, which appears highly hypomethylated, clearly indicates that the predicted promoter
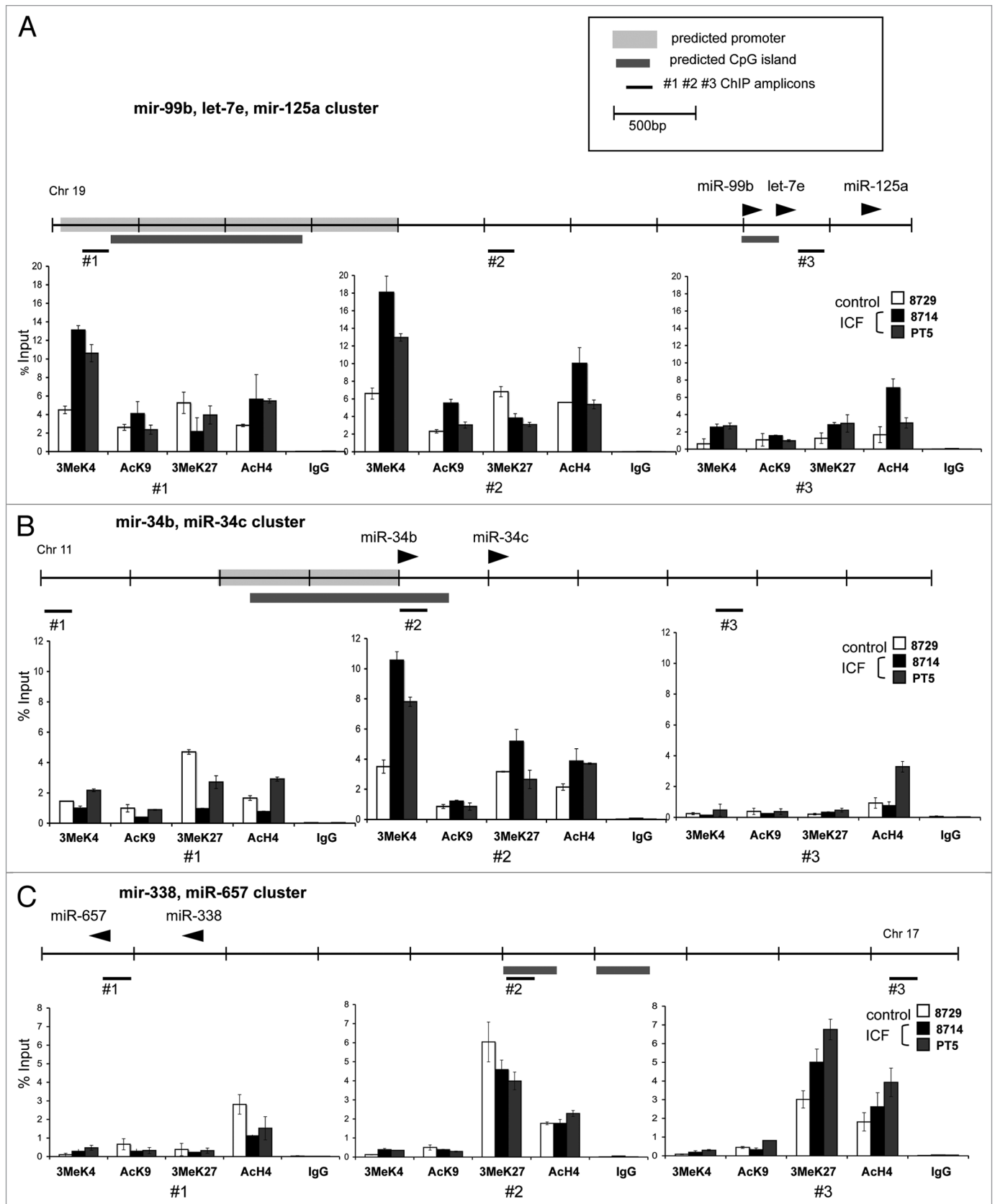
Figure 4. (A–C) For figure legend see page 439..
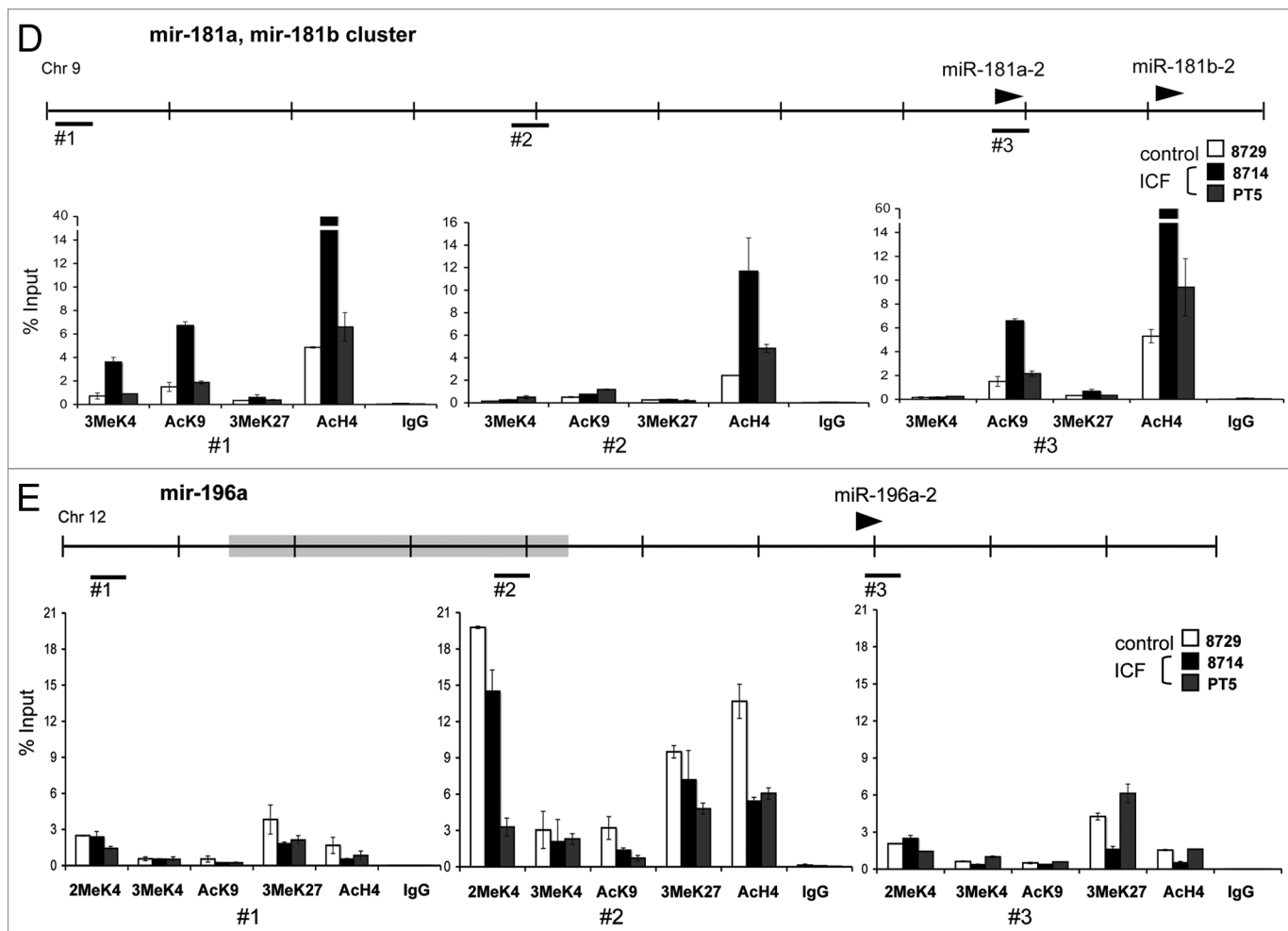
Figure 4. Chromatin immunoprecipitation (ChIP) analysis of four upregulated microRNA clusters and one downregulated microRNA in ICF LCLs. #1, #2 and #3 represent amplicons analyzed and their relative positions to the microRNA clusters and to CpG-rich regions are shown. Antibodies against H3 di or trimethylated on lysine 4 (2- or 3meK4), H3 acetylated on lysine 9 (AcK9), histone H4 acetylated (AcH4) and H3 trimethylated on lysine 27, (3meK27) have been used in each experiment, together with rabbit IgG as background control. 8714 and PT5 ICF cells were compared to 8729 control cells. Real Time PCR of immunoprecipitated material has been performed and % of input for each sample is reported in the graph. Reactions have been repeated at least three times from two independent ChIP experiments.

could effectively function in controlling the transcriptional status of all three microRNAs within the cluster.

We next examined mir-34b/c cluster that was upregulated in ICF cells (**Fig. 4B**). Three regions have been analyzed by ChIP assay, two upstream and one downstream of the mature microRNAs sequences. Both ICF LCLs 8714 and PT5 exhibited increased K4H3 trimethylation and histone H4 acetylation within the region #2 spanning both microRNAs. However, a reduction of K27H3 trimethylation mark seems to be restricted only to region #1, and not to region #2, where the level of this histone isoform did not change, and in one of the ICF samples is even slightly increased. The combination of both repressive and active histone modification at the same region has been previously reported for some genes in T cells responding to the need to be readily expressed or silenced.[37]

While H4 acetylation level remained relatively high, the differential enrichment of K4H3 trimethylation mark was completely lost in the downstream region, indicating that the putative regulatory sequences of this cluster could be restricted within the 2 kb upstream of the two microRNAs, miR-34b and miR-34c.

We also observed the concomitant permissive H4 acetylation and repressive K27H3 trimethylation marks when examining miR-338/657 cluster, which shows overexpression in all two ICF LCLs. As demonstrated in **Figure 4C**, the region closer to the microRNAs sequences did not exhibit any differential histone profile, whereas the farther regions #2 and #3 had H4 acetylation and K27H3 trimethylation enriched in normal and ICF samples. It appears that K27H3 trimethylation becomes inversely enriched going from region #2 to the region #3, suggesting that this histone mark may have a regulative role.

To extend our ChIP analysis to dysregulated microRNAs possibly contributing to ICF phenotype, we determined the histone marks profile of miR-181a/b cluster and miR-196a,

respectively up and downregulated in ICF samples (**Fig. 4D and E**). In both cases, although the same microRNAs are localized on two chromosomes, we only observed differential histone enrichments at the level of one microRNA locus.

H4 acetylation is the only histone mark corresponding to permissive chromatin markedly increased in ICF samples alongside the three examined regions #1, #2 and #3 of miR-181a/b cluster on chromosome 9, whereas the di- or trimethylK4 and acetylK9 were only slightly higher in ICF samples compared to controls. K27H3 trimethylation was almost unchanged.

As for miR-196a, the region 2 kb upstream of the microRNA sequence appears to show the most drastic reduction of dimethyl-K4 and acetyl-H4, in accordance with the silencing of this microRNA in ICF samples compared to wild-type. Regarding the trimethylated isoform of K4H3 no difference was observed.

Finally, we examined histone marks of those microRNAs detected only in ICF LCLs and fibroblasts (miR-203) or only in ICF LCLs (miR-153, miR-369) compared to controls, whose methylation status was previously analyzed (**Suppl. Fig. 2A–D**). Again we found slight changes of AcH4 and 3meK4H3 in ICF samples at genomic region surrounding miRNAs especially analyzing LCLs.

Taken together, ChIP analysis revealed that most microRNAs upregulated in ICF cells show reduction in histone modifications, which are characteristic of repressed chromatin, such as histone H3K27 trimethylation. Conversely, it revealed an increase in modifications, which are characteristic of transcriptionally active chromatin, particularly in histone H3K4 trimethylation and H4 acetylation. The differential enrichment profile appears to be limited to regions upstream and/or spanning microRNA mature sequences, suggesting that it is critical for microRNA transcriptional control.

Discussion

The results of our studies presented here provide a better understanding of the interaction between DNA methylation and histone code in regulating microRNA genes and enable us to predict some molecular events possibly contributing to ICF phenotype.

The strict interplay between DNA methylation and histone modifications in regulating gene expression is well documented and the direct interaction between chromatin modifying proteins has been demonstrated in several cell contexts. However, the precise nature of their mechanistic relationship and how the epigenetic mechanisms can influence each other remain largely obscure. Even less it is known about the microRNAs transcription programs.

Increasing evidence indicates that a substantial number of microRNA genes are subjected to epigenetic modification and, on the other hand, it is emerging that microRNAs themselves can regulate chromatin modifiers. A conspicuous number of CpG islands-associated microRNAs has been found highly hypermethylated in cancer cells, indicating that altered miRNA gene methylation might contribute to human tumorigenesis. Indeed, the increased expression of these genes, following the 5-azacytidine treatment, has confirmed the pivotal role of this epigenetic mechanism in microRNAs transcriptional control.[38-40]

The human ICF syndrome provided us the opportunity to investigate the effect of the deficiency of one of the main player of epigenetic network, the DNA methyl-transferase 3B, on microRNA genes expression regulation and the influence on the other epigenetic factors involved. We identified approximately 100 microRNA genes with an aberrant expression pattern in ICF relative to normal cells. More than one half of identified microRNAs were associated to CpG islands, thus representing candidate targets of the DNA methylation machinery. However, DNA methylation level was not significantly reduced at microRNA cluster examined, as direct consequence of DNMT3B loss of function.

Two specific CpG dinucleotides within the miR-34b/c cluster CpG island, previously characterized and shown to be hypermethylated in cancer cells,[21] showed reduced DNA methylation in ICF cells compared with one normal cell line, but not with the other two control cell lines.

As the differential methylation appears to be limited to specific CpGs, we cannot exclude the possibility to have missed the regions target of DNMT3B activity of the other microRNA clusters chosen in our bisulfite analysis. This is consistent with the fact that most CpG-rich regions associated with microRNA genes are only predicted to have a regulatory role and that, even more importantly, the position of transcription start sites of the microRNA primary transcripts is mostly unknown. However, the possibility of an indirect effect of DNMT3B deficiency, mediated by dysregulated transcription factors that, in turn, control the expression of a certain number of microRNAs, appears the most plausible, thus explaining the failure to find extensive changes in DNA methylation in ICF cells. Similar observations in cancer cells, where Dnmt3B gene targeting results in cases of genes reactivation not associated with promoter hypomethylation also support this hypothesis.[41]

We cannot exclude that at least two microRNA clusters analyzed, miR-338/657 and miR-199a/a*, both being intragenic, could undergo the same transcriptional regulation of their overlapping transcripts, including a putative DNA methylation mediated control of their 5' UTRs. However, the two known genes hosting these upregulated microRNAs are not differentially expressed in ICF samples compared with normal cells, suggesting that microRNAs are independently transcribed and that CpG islands of these genes do not control microRNAs expression.

Despite the DNA methylation profile, there appear to be more significant changes in the histone modification patterns following ChIP analysis of four upregulated microRNA clusters and one downregulated microRNA in ICF syndrome cells. This revealed specific changes in levels of histone modifications, particularly at H4 acetylation, K27H3 trimethylation and K4H3 trimethylation that were mostly consistent with the altered expression of these molecules. Some of the histone marks similarly change in both ICF samples, as for 3meK4H3 at regions #1 and #2 of miR-99b and region #2 of miR-34b/c or AcH4 at region #2 of miR-196. Nevertheless, we report variability between the two

patients in most cases, being the value of the enrichment of several histone isoforms quite different.

Intriguingly, we observed that complete loss and/or gain of a specific histone mark never occurred at any examined region comparing ICF cells with control cells. On the contrary, it appears that the ratios of active and inactive signals, corresponding to opposite histone marks, are the critical factors associated with chromatin activity. This finding, which has been previously supposed to be important to modulate gene expression in T cells,[37] could be similarly involved in the regulation of microRNAs expression levels.

We also observed the coexistence of two apparently opposing marks H3K27 trimethylation and H4 acetylation in the region farther away from microRNAs 338 and 657, both in ICF and control cells. A subpopulation of chromatin regions at certain genes in T cells is associated with both permissive and repressive histone marks, particularly H3K4 trimethylation and H3K27. However, colocalization of H3K27 trimethylation and acetylated histone H4 has not been tested.[37]

Overall, these studies further emphasize that DNA methylation and histone modifications are strictly interrelated and that DNMT3B is able to repress transcription independently from de novo methylation activity.

It is known that DNA methyltransferases are able to directly or indirectly interact with several elements of the chromatin modification machinery, such as HDACs 1 and 2, SUV39H1 and hSNF2H, as well as the machinery regulating chromosome structure at a genome-wide level (hCAP-C and hCAP-E, or the condensin complex).[42,43]

Mutations in Dnmt3B may directly affect the chromatin-modifying functions of remodelling complexes by altering their recruitment or their ability to set active or inactive chromatin states at gene level. Beside a local effect on microRNA promoters and/or regulatory regions, loss of DNMT3B function could indirectly impair the activity of the proteins modulating chromatin structure, through the disruption of pericentromeric heterochromatin, where these complexes localize in the nucleus.

In addition, hypomethylation of satellite DNA at pericentromeric heterochromatin might alter the nuclear compartmentalization of microRNA-containing genomic region or segregation of certain transcription regulatory proteins that can bind specific heterochromatin sequences as well as microRNA promoters.

Centromeric heterochromatin can exert such indirect effects during murine lymphogenesis on several genes and this is mediated by Ikaros, a transcription regulatory factor that specifically binds the promoters of these genes and centromeric heterochromatin.[44-46] Different heterochromatin domains at chromosome 1, 16 and 9, aberrantly altered in ICF syndrome might act independently to control transcription in trans of different small portions of the human genome, containing genes and microRNAs. Moreover, as our recent finding proved that some misexpressed genes in ICF LCLs show an altered localization within the X and Y chromosome territories, compared to control cells, we could expect that there is an impaired regulatory effect in trans of higher order nuclear organization.[47,48]

However, whatever mechanism is responsible for microRNA up and/or downregulation events, our data are consistent with results described in Jin et al. 2008, in which altered gene expression profiles are mainly associated to changes in histone modification marks rather than to a marked promoter hypomethylation. Thus, microRNA-codifying genes appear to behave as classical protein-codifying genes in terms of response to Dnmt3B mutations.

Owing to the rarity of ICF syndrome, we have been limited to use immortalized LCLs for our studies. Thus, we cannot prove that misregulation of the microRNAs we have studied in LCLs also occurs in the tissue where they could exert their primary effects. Such a confirmation will surely require an extensive examination of the mouse model of ICF syndrome. Nonetheless, our results also represent a step forward to better understand the molecular defects that give rise to the ICF syndrome phenotype. This is relevant because even if ICF syndrome has been described many years ago, its pathogenesis is far from being elucidated.

The bioinformatic analysis indeed suggest that misregulation of specific microRNAs may be responsible for certain aspects of immune defects as well as those related to neural function and development. For instance, among dysregulated microRNAs, we identified important regulators of B and T cells activation and differentiation, such as miR-181a, miR-181b and miR-125, whose validated target genes are downregulated. This represents the first step of a new functional approach of study in order to unravel the complex and intricate molecular riddle of ICF syndrome.

Materials and Methods

Cell lines and patients. ICF syndrome cell lines used in this study include Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs) GM08714 and PT5 and fibroblast cell lines GM08747 and PT3. GM08714 and GM08747 are cells from the same heterozygous ICF patient P4 [A603T and intron 22 G to A mutation resulting in insertion of three amino acids (STP) in DNMT3B]; PT5 is the LCL derived from the heterozygous patient PT5 (V699G and R54X); PT3 is a fibroblast cell line derived from the homozygous patient PT3 (STP807ins). Control cells include LCLs derived from normal individuals, GM08728 and GM08729, and the WI38 fibroblast cell line.

LCLs were grown in RPMI1640 media (Euroclone) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (Euroclone). Fibroblasts were grown in MEM medium supplemented with 2 mM L-glutamine and 15% fetal bovine serum.

Microarray hybridizations and data processing. For each sample, technical replicates were produced and total RNA was hybridized for 30 min at 60°C and 18 h at 45°C on *Human MicroRNA Sentrix Universal-96 Array Matrix (SAM),* according to the protocol provided by the manufacturer (Illumina Inc., San Diego, CA, USA). In short, 200 ng of total RNA from each sample is polyadenylated with a poly-A polymerase[49] (PAP) enzyme; polyadenylated RNA is then converted to cDNA using a biotinylated oligo-dT primer with a universal PCR sequence followed by hybridization with the set of miRNA-specific

oligos (MSO). These MSOs are extended and added flurophore followed by hybridization to beads on the Sentrix Array Matrix. The array matrix was scanned using Illumina BeadArray Reader 500, which measures fluorescence intensity of the signal that corresponds to the quantity of the miRNA in the original sample.

For data analysis, the intensity files were loaded into the Illumina BeadStudio v3.3 software for quality control and expression analysis.

First, the average normalization algorithm was applied on the dataset to correct systematic errors. Average normalization is a method where the sample intensities are scaled by a factor equal to the ratio of average intensity of virtual sample to the average intensity of the given sample.

For differential expression analysis, technical replicates of each sample were grouped together and miRNAs with a detection p-value below 0.05, corresponding to a false positive rate of 5%, were considered as detected. Differently expressed miRNAs were selected with DiffScore cut-off set at $\pm|20|$, with a p value ≤ 0.01.

Quantitative real time PCR of miRNAs. MicroRNA expression was measured using the TaqMan microRNA quantitative PCR (Applied Biosystems, Rotterdam, The Netherlands) with the Applied Biosystems Real Time Q-PCR ABI Prism 7900HT. Briefly, 10 ng of total RNA was reverse transcribed using the MicroRNA reverse transcription kit (Applied Biosystems) and a specific reverse transcription stem-loop primer, according to the manufacturer's protocol. All reactions were run in duplicate and the expression of the endogenous control, RNU44 (purchased from Applied Biosystems), was measured under the same conditions in all samples. The expression of each microRNA relative to RNU44 was determined using the $2^{-\Delta\Delta Ct}$ method. MicroRNA levels are expressed in fold change of the target miR expression.

Bisulfite genomic sequencing (BGS). Bisulfite genomic sequencing was performed according to Epitect Bisulfite kit instruction (Qiagen). For a complete listing of PCR primer sequences used for BGS, refer to **Supplementary Table 1**. Amplified DNA fragments were extracted from agarose gel and cloned by using TA cloning kit (Invitrogen, Carlsbad, CA, USA).

Sequencing of clones has been performed by using M13 forward and reverse primers (Primm Biotech).

Chromatin immunoprecipitation (ChIP). ChIP has been essentially performed as previously described.[48] Briefly, for ChIP, 1×10^7 cells were fixed with 1% formaldehyde. After cross-linking, chromatin was isolated and subjected to sonication, resulting in 200–1,000 bp DNA fragments. After immunoprecipitation with anti-acetylated and anti-methylated histone H3- and H4-specific antibodies (Upstate Biotech), immunocomplexes were isolated by co-precipitation with protein A-Sepharose (Pharmacia). Anti-IgG was used as negative control. The recovered DNA was measured and the quantitation of cromatin-immunoprecipitated DNA fragments was performed by quantitative Real Time PCR, by using SYBR Green quantitative PCR (iQ SYBR Green Supermix, Biorad) performed according to CFX96™ Real Time PCR Detection Systems. The enrichment of DNA was calculated in terms of % input = $2^{-\Delta Ct} \times 100$, where ΔCt (threshold cycle) is determined by $Ct_{IP\,sample} - Ct_{Input}$ and 100 refers to the input being 1% of the chromatin amount exposed to IP.

MiRNA target prediction and functional analysis of predicted mRNA targets of the miRNAs. MAMI (http:/mami.med.harvard.edu) was used to predict targets by using its accuracy optimization settings. MAMI has a compilation of target prediction tools, DIANA-microT,[49] miRanda,[50] TargetScanS,[51] miRtarget[52] and PicTar.[53] The miRNAs are based on miRs registry version 8.1 (5/06).

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/GattoEPI5-5-Sup.pdf

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