Iran. J. Biotechnol. January 2023;21(1): e 3211 DOI: 10.30498/ijb.2022.317078.3211

Research Article

The PBX1/miR-141-miR-200a/EGR2/SOCS3 Axis; Integrative Analysis of Interaction Networks to Discover the Possible Mechanism of MiR-141 and MiR-200a-Mediated Th17 Cell Differentiation

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Received: 2021/11/28; **Accepted:** 2022/03/06

Background: Overexpression of miR-141 and miR-200a is known to be associated with the differentiation of T helper 17 (Th17) cells, which are key players in the pathophysiology of autoimmune disorders. However, the function and governing mechanism of these two microRNAs (miRNAs) in Th17 cell skewing are poorly defined.

Objectives: The aim of the present study was to identify the common upstream transcription factors and downstream target genes of miR-141 and miR-200a to obtain a better insight into the possible dysregulated molecular regulatory networks driving miR-141/miR-200a-mediated Th17 cell development.

Materials and Methods: A consensus-based prediction strategy was applied for *in-silico* identification of potential transcription factors and putative gene targets of miR-141 and miR-200a. Thereafter, we analyzed the expression patterns of candidate transcription factors and target genes during human Th17 cell differentiation by quantitative real-time PCR and examined the direct interaction between both miRNAs and their potential target sequences using dual-luciferase reporter assays.

Results: According to our miRNA-based and gene-based interaction network analyses, *pre-B cell leukemia homeobox 1* (*PBX1*) and *early growth response 2* (*EGR2*) were respectively taken into account as the potential upstream transcription factor and downstream target gene of miR-141 and miR-200a. There was a significant overexpression of the *PBX1* gene during the Th17 cell induction period. Furthermore, both miRNAs could directly target *EGR2* and inhibit its expression. As a downstream gene of *EGR2*, the *suppressor of cytokine signaling 3* (*SOCS3*) was also downregulated during the differentiation process.

Conclusions: These results indicate that activation of the PBX1/miR-141-miR-200a/EGR2/SOCS3 axis may promote Th17 cell development and, therefore, trigger or exacerbate Th17-mediated autoimmunity.

Keywords: Autoimmunity, EGR2, MiR-141, MiR-200a, PBX1, T helper 17 cells

1. Background

Autoimmunity is defined as the failure distinguishing of self-antigens from non-self and subsequent mistaken immune responses of an organism against its own normal body components (1). Large-scale epidemiological studies have reported that 3-5% of the human population is affected by at least one of more than 100 different autoimmune diseases and the prevalence is rising alarmingly by as much as 9% each year (2). The development of an autoimmune condition requires genetic deficiencies, environmental factors and inappropriate immune cells development, all of which trigger autoreactive immune responses that lead, ultimately, to the destruction of healthy tissues (3).

In the context of aberrant immune cells development, it was traditionally supposed that autoimmune conditions were mainly related to dysregulated T helper (Th) 1 cell response. However, subsequent studies revealed that Th17 cells are major initiators and participants in the pathogenesis of autoimmune disorders (4). Th17 cells represent a distinct subset of $CD4^+$ T cells that are differentiated from naïve T cells in the presence of interleukin (IL) -6, IL-1, IL-21 and IL-23 (5). Th17 cell skewing is regulated by lineage-defining transcription factors of STAT3/RORγt/Batf and leads to the secretion of highly pro-inflammatory cytokines including IL-17A and IL-17F (6). Despite significant advances in defining Th17 cell function in autoimmunity, more scientific discoveries are needed to characterize novel regulating factors involved in Th17 cell development. Identification of such regulatory molecules could be reflected in the futuristic therapies that moderate Th17-related autoimmune inflammatory responses.

microRNAs (miRNAs), a class of single-stranded non-coding RNAs, have been reported to serve as key gene regulators in both physiological and pathological states. Through binding to the 3´untranslated region (UTR) of their target messenger RNA (mRNA), miRNAs are capable of post-transcriptional gene silencing (7). In the immune system, it has been estimated that more than 500 different miRNAs are expressed in T cells and their expression pattern is strictly time-dependent (8). This is notable as a deregulated expression of these small RNAs during T cell differentiation can result in immune dysfunction or autoimmune conditions. The miR-200 family consists of five different miRNAs, namely miR-200a, miR-200b, miR-200c, miR-429 and miR-141. Although miR-141 and miR-200a are located in different genomic regions, they belong to a distinct functional group from other members since they share the same seed sequence and are likely to silence myriad common target genes (9). Accruing research has identified various factors involved in miRNAs biogenesis and function, but little is known about the upstream regulation of these non-coding RNAs. Similar to the regulation of protein-coding genes, transcription factors (TFs) are thought to control the expression of miRNAs through binding to the regulatory sequences located upstream of their genes (10, 11).

As a transcription factor, pre-B cell leukemia homeobox1 (PBX1) regulates chromatin accessibility and therefore expression level of its downstream genes (12). Recent data has demonstrated that PBX1 is more frequent in T cells from lupus patients than from healthy volunteers (13). In addition, transgenic expression of *PBX1* gene in $CD4^+$ T cells upregulated the expression of miR-155, miR-10a and miR-21, which resulted in an impaired generation of regulatory T (Treg) cells and increased frequency of follicular helper T (Tfh) cells (13). Although PBX1 plays a role in the development of autoreactive Tfh lineage and thereby in autoimmune pathology, questions about its probable inducing effects on the Th17-mediated autoimmunity remain unanswered. On the other hand, early growth response 2 (EGR2) is a zinc-finger transcription factor that serves as a unique negative modulator of Th17 cell differentiation (14). Direct upregulation of the *suppressor of cytokine signaling 3* (*SOCS3*) gene by EGR2 is supposed to be vital for limiting Th17 cell development and preventing its associated autoimmune reactivity (15).

2. Objectives

Based on our earlier data, dysregulated expression of miR-141 and miR-200a is implicated in Th17 cell differentiation (16). However, the reasons for deregulated expression of these two miRNAs as well as the molecular mechanisms that direct Th17 cell skewing following miR-141/miR-200a upregulation are not fully deciphered. Therefore, after integrating computational predictions from different data types, we sought to determine if PBX1 functions in Th17 cell differentiation through a pathway that involves miR-141 and miR-200a upregulation. Furthermore, we planned to explore the direct interface between miR-141/miR-200a and *EGR2*, as a potential target gene of both miRNAs, and evaluate the expression profiles of this Th17 cell inhibitor and its downstream *SOCS3*

gene to investigate the possible mechanism of miR-141/miR-200a-induced Th17 differentiation.

3. Materials and Methods

3.1. MiRNA-Based Regulatory Network Construction

Three online bioinformatic databases including miRDB (http://mirdb.org/), microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) and TargetScan (http://www.targetscan.org/) that consist of computationally predicted miRNA-target interactions, were used to identify potential target sequences of miR-141 and miR-200a in the human genome. Predicted targets from miRDB with a target score ≥ 80, microT-CDS with a miTG score ≥ 0.7 and TargetScan with a context++ score percentile ≥ 80 were imported to Venn's diagrams drawing tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) to only retain the common potential targets for each miRNA (17, 18). These strict prediction cutoffs were employed to minimize false-positive selections and retrieve more confident target genes. Subsequently, transcription factors regulating the gene expression of miR-141 and miR-200a were obtained from the TansmiR database (http://www.cuilab.cn/transmir). We only retrieved more stringent-evidence level transcription factors and then investigated the protein expression of final candidates in lymphoid organs using The Human Protein Atlas (https://www.proteinatlas.org/). We constructed a regulatory network that included miR-141 and miR-200a, their potential upstream transcription factors and their predicted downstream target genes. Cytoscape software (https://cytoscape.org/) was used for network data integration and visualization.

3.2. DNA Sequence Analysis

Using DNA sequence analysis, the potential regulation of miR-141 and miR-200a by the candidate transcription factor was evaluated. 5000 bp DNA sequence upstream and 1000 bp spanning sequence downstream of the transcription start site of miR-141 and miR-200a were obtained from UCSC (https://genome.ucsc.edu/) and miRBase (http://www.mirbase.org/). Transcription factor binding sites were predicted using the JASPAR database (https://jaspar.genereg.net/) and the possible interactions between miR-141/miR-200a regulatory sequences and the candidate transcription factor were examined through rVista online database (https://rvista.dcode.org/) and hTFtarget tool (http://bioinfo.life.hust.edu.cn/hTFtarget/).

3.3. MiRNA Target Analysis

To explore more confident target genes for miR-141 and miR-200a, three lists of predicted targets were obtained from miRDB, microT-CDS and TargetScan databases based on selected cutoff criteria explained in the step 3.1. The fourth list included 115 genes robustly associated with the Th17 cell differentiation (19). Using Venn diagrams, a common-target screening strategy was conducted to retrieve high-scoring target genes which are affected by both miRNAs and also implicated in the Th17 cell development. Validated miRNA-target pairs with strong experimental methods including western blot, qPCR and reporter assay were identified using TarBase (https://carolina.imis.athena-innovation.gr/diana_tools/) and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) and excluded from final candidate target genes.

3.4. Gene-Based Interaction Network Construction

In order to accomplish a gene interaction analysis for the potential target gene of miR-141/miR-200a, we constructed a gene-based interaction network that contains two sets of important genes involved in autoimmune disorders or Th17 cell differentiation. Autoimmunity-related and Th17-associated genes were respectively recruited from the DisGeNET database (http://www.disgenet.org/) with a gda score >0.2 and our earlier work (19, 20). We performed the gene interaction analysis using the STRING database (https://string-db.org/) and visualized the significant interactions (Interaction Score > 0.7) via Cytoscape software (https://cytoscape.org/).

3.5. Molecular Signaling Pathway Enrichment Analysis

Enrichr, an enrichment analysis web-based tool (https://maayanlab.cloud/Enrichr/), was utilized to find the most related signaling pathways to the potential target gene of miR-141/miR-200a and its top 100 co-expressed genes identified with ARCHS4 RNA-seq gene-gene co-expression matrix. The significantly enriched KEGG, PANTHER and REACTOME pathways (p<0.05) were extracted from the Enrichr and summarized using its plug-in software application, Appyters (https://appyters.maayanlab.cloud/).

3.6. Th17 Cell Differentiation

For Th17 cell differentiation, naïve $CD4^+$ T cells were isolated from fresh human peripheral blood mononuclear cells (hPBMCs) with the help of Naïve $CD4^+$ T Cell Isolation Kit II which is designed based on a magnetic cell sorting system (MACS) (MiltenyBiotec, Bergisch Gladbach, Germany). Subsequently, the obtained naïve T cells were seeded in a 12-well plate with the Th17 cell polarizing medium prepared according to the CellXVivo Human Th17 Cell Differentiation Kit manufacturer's instruction (R&D Systems, Minnesota, USA). The induction process was continued for duration of six days and the differentiation medium was refreshed every other day.

3.7. Enzyme-Linked Immunosorbent Assay (ELISA)

Quantikine ELISA Human IL-17 Immunoassay Kit (R&D, Minnesota, USA) was utilized according to the manufacturer's protocol to measure the amount of IL-17A cytokine secreted by differentiating cells after 5 hours and 2 days, 4 days and 6 days of the induction period. All samples were measured in duplicate at 450 nm.

3.8. Quantitative Real-Time PCR (qRT-PCR)

To assess gene expression level, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA). Next, one microgram of total RNA was reverse transcribed into the complementary DNA (cDNA) by RevertAid First Strand cDNA synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using random hexamer primers. Finally, qRT-PCR was performed in triplicate with the specific gene primers (**Supplementary Table 1**) on a Step One Plus Real-Time PCR thermal cycler (Applied Biosystems, Foster City, USA) using SYBR Premix Ex *Taq*II (TaKaRa, Kyoto, Japan) in accordance with the manufacturer's instructions. Relative expression of mRNAs was evaluated by the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of *ß-actin* as the reference gene.

3.9. Plasmid Construction and Dual-Luciferase Reporter Assay

hsa-miR-141 and hsa-miR-200a precursor DNA sequences as well as their 250 bp spanning upstream and downstream sequences were obtained from the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/) to design their specific primers (**Supplementary Table 2**). The 500 bp spanning precursor of each miRNA was amplified using PCR and the purified PCR products were then inserted into the digested p-Bud-EGFP vectors (Promega, Madison, USA). For the binding site mutation assay, the possible binding site of miR-141/miR-200a on their candidate target gene was predicted by the Targets can database (http://www.targetscan.org). The residues within the predicted binding site were replaced with a thymidine heptamer to generate mutant oligomers (**Supplementary Table 2**). Next, synthesized fragments of wild-type (WT) and mutant (MUT) target sites were introduced into the psiCHECK2 vectors (Promega, Madison, USA). These luciferase reporter plasmids were co-transfected with either recombinant or empty (negative control) p-Bud-EGFP vectors into HEK293T cells and cultured for a duration of 48 h. Luciferase activity was assessed using a Dual-Luciferase Reporter Assay Kit on a Glomax Luminometer fluorescence detector (Promega, Madison, USA). The luminescent signal, which reflects the expression level of the target reporter gene, was calculated using the ratio of the Renilla relative light units (RLU) to the firefly RLU.

3.10. Statistical Analysis

All statistical tests were executed by GraphPad Prism 8 software. Data are presented as mean ± standard error of the mean (SEM). Comparisons between two groups were analyzed using the unpaired t-test and a value of $p<0.05$ was considered significant.

4. Results

4.1. Interaction Analysis of MiRNAs-Target Genes

To include maximum predicting algorithms with different target scoring parameters, three different miRNA target prediction databases were employed. In this context, predicted and validated target genes of miR-141 and miR-200a were identified using miRDB, microT-CDS and TargetScan databases under the aforementioned prediction thresholds (Supplementary Excel files 1 and 2, sheet 1).

Furthermore, Venn diagrams were drawn for common targets between these three databases for each miRNA **(Fig. 1A ,1B**). Totally, we identified 192 and 214 miRNA-target interactions for miR-141 and miR-200a, respectively (**Supplementary Excel files 1 and 2, sheet 2**).

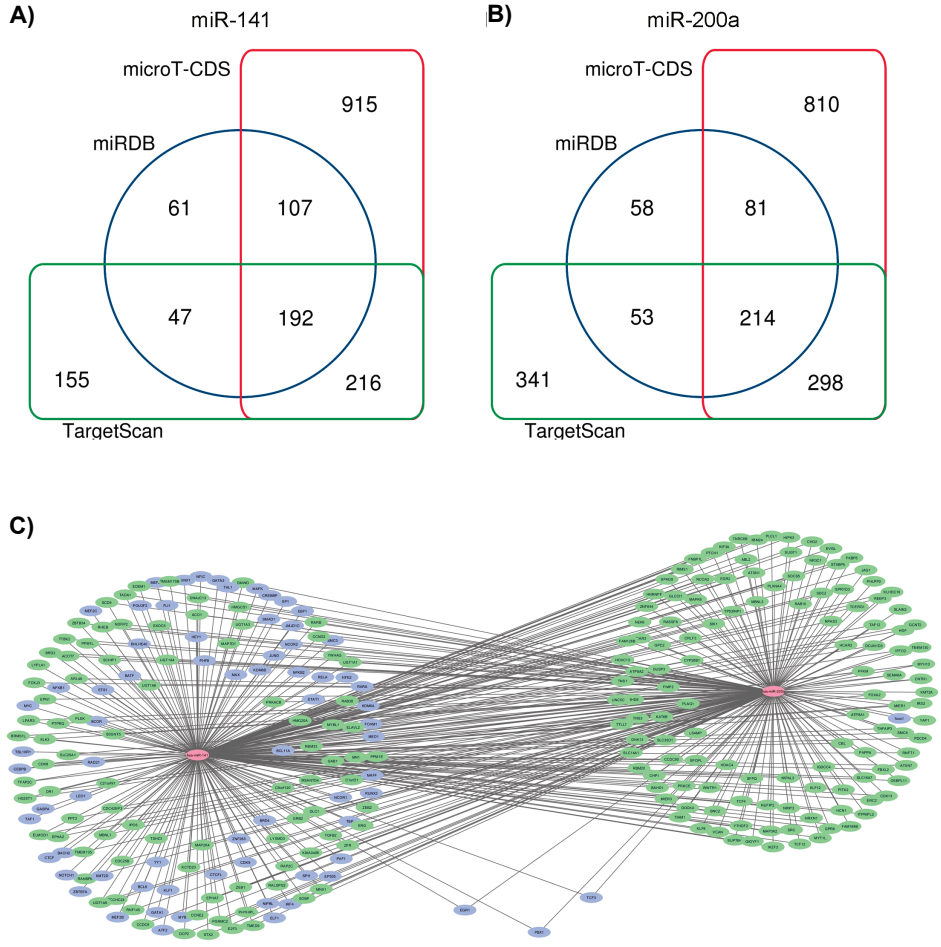


Figure 1. miRNA-based regulatory network. (A, B) Common target genes of each miRNA (miR-141 and miR-200a) from miRDB, microT-CDS and TargetScan databases were identified using Venn diagrams. **C)** The miRNA-based regulatory network visualized via Cytoscape depicted 3 common TFs and 184 common target genes for both miRNAs. The pink nodes represent miRNAs, the blue nodes represent TFs and the green nodes represent target genes.

4.2. Regulation Analysis of Transcription Factors-MiRNAs

To investigate the possible underlying mechanism of aberrant regulation of miR-141 and miR-200a during Th17 cell differentiation, we first explored the upstream transcription factors of these two miRNAs. Using TransmiR, a database for experimentally supported and predicted TF-miRNA regulations, we found 74 TFs for miR-141 and a mere of 4 TFs for miR-200a (**Supplementary Excel file 3**). To achieve more reliable results in our subsequent analyses of TF-miRNA interactions, we only retrieved literature-derived regulations and more stringent TFs predicted from ChIP-seq evidence.

4.3. MiRNA-Based Regulatory Network Analysis Revealed Putative Common Transcription Factors of MiR-141 and MiR-200a

We constructed a regulatory network that included our miRNAs, their putative upstream transcription factors and predicted downstream target gens, using information obtained from our previous step *in- silico* analyses and with the help of Cytoscape software (**Fig. 1C**). Cytoscape integrated and visualized the interaction between 486 nodes (2 miRNAs, 78 transcription factors and 406 target genes); therefore, we could separate common TFs and target genes of miR-141 and miR-200a. Overall, our miRNA-based regulatory network depicted three common predicted TFs, namely PBX1,

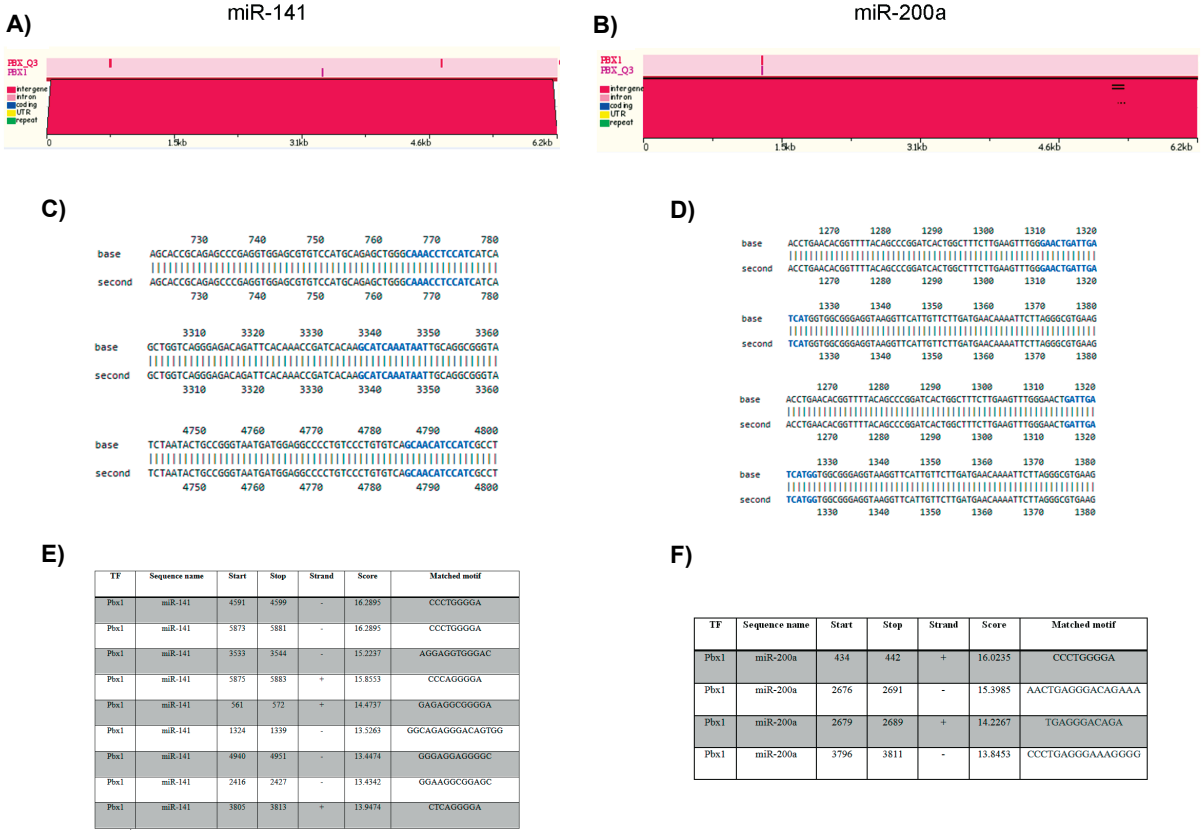
Egr1 and Tcf3, among which PBX1 is only supposed to contribute positively to miR-141/miR-200a expression and therefore was selected as the candidate upstream TF for subsequent investigations. Using this regulatory network analysis, we also determined 184 common target genes of both miRNAs and, hence, performed a more purposeful strategy (explained in step 4.5) to extract a shorter list of potential targets.

4.4. DNA Sequence Analysis Predicted PBX1 Interaction with Specific Sites in the Regulatory Elements of MiR-141 and MiR-200a Genes

To explore the possible regulatory effect of our candidate TF on miR-141 and miR-200a expression, we scanned 6 kb DNA sequence surrounding the transcription start site of each miRNA for 18 PBX1 binding sites obtained from the JASPAR open-access database (**Supplementary Word file 1**). Our analysis in the rVista database identified three and two PBX1 binding sites respectively on miR-141 and miR-200a regulatory elements (**Fig. 2A-2D**). Interestingly, several PBX1 binding motifs located within the designated miR-141 and miR-200a promoter sequence were also predicted using the hTFtarget database (**Fig. 2E, 2F**). Collectively, these data demonstrate the feasibility of PBX1 interaction with different response elements within the regulatory region of miR-141 and miR-200a genes.

4.5. MiR-141/miR-200a Target Gene Analysis Identified EGR2 as their Potential Downstream Target

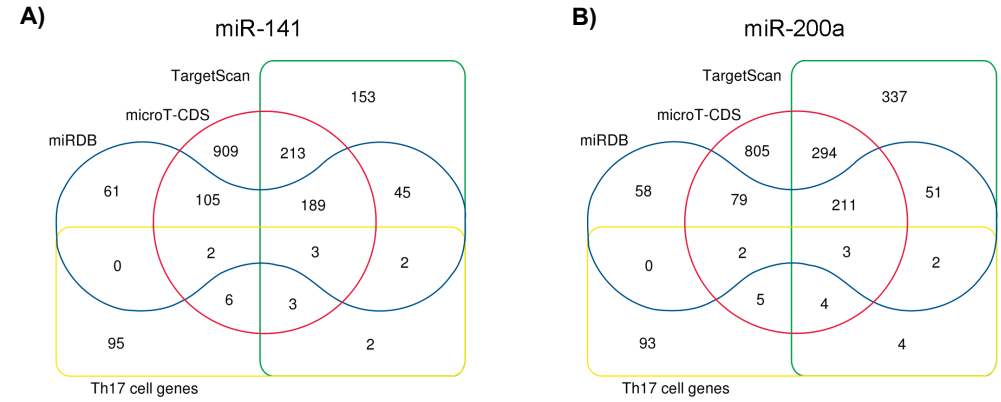
To explore the common target genes of miR-141 and miR-200a in the Th17 cell differentiation process, we imported four different lists of target genes (Three lists from miRDB, microR-CDS and TargetScan databases, and one list of Th17 cell-associated genes)



TF	Sequence name	Start	Stop	Strand	Score	Matched motif
Pbx1	miR-141	4591	4599	-	16.2895	CCCTGGGGA
Pbx1	miR-141	5873	5881	-	16.2895	CCCTGGGGA
Pbx1	miR-141	3533	3544	-	15.2237	AGGAGGTGGGAC
Pbx1	miR-141	5875	5883	+	15.8553	CCCAGGGGA
Pbx1	miR-141	561	572	+	14.4737	GAGAGGCGGGGA
Pbx1	miR-141	1324	1339	-	13.5263	GGCAGAGGGACAGTGG
Pbx1	miR-141	4940	4951	-	13.4474	GGGAGGAGGGGC
Pbx1	miR-141	2416	2427	-	13.4342	GGAAGGCGGAGC
Pbx1	miR-141	3805	3813	+	13.9474	CTCAGGGGA

TF	Sequence name	Start	Stop	Strand	Score	Matched motif
Pbx1	miR-200a	434	442	+	16.0235	CCCTGGGGA
Pbx1	miR-200a	2676	2691	-	15.3985	AACTGAGGGACAGAAA
Pbx1	miR-200a	2679	2689	+	14.2267	TGAGGGACAGA
Pbx1	miR-200a	3796	3811	-	13.8453	CCCTGAGGGAAAGGGG

Figure 2. DNA sequence analysis. (A, B) Using rVista database, three and two PBX1 binding sites were, respectively, identified on miR-141 and miR-200a promoters. **(C, D)** DNA sequence of predicted PBX1 binding sites on miR-141 and miR-200a regulatory elements are shown in blue characters. **(E, F)** significant interactions of PBX1 with miR-141 and miR-200a predicted via hTFtarget database.



C)

		miRDB	microT-CDS	TargetScan
Gene	microRNA	Target Score	miTG Score	Context++Score Percentile
EGR2	hsa-miR-141	82	0.701	81
	hsa-miR-200a	82	0.702	81

D)

Position 1029-1035 EGR2 3′UTR
hsa-miR-141
5' ...GUUUUGUGACUUAAAAGUGUUAC
3' GGUAGAAAUGGUCUGUCACAAU

Position 1029-1035 EGR2 3′UTR
hsa-miR-200a
5' ...GUUUUGUGACUUAAAAGUGUUAC...
3′ UGUAGCAAUGGUCUGUCACAAU

Figure 3. miRNA-target gene analysis. (A, B) Venn daigrams showed a complete overlap in common target genes of miR-141 and miR-200a from miRDB, microT-CDS, TargetScan and Th17-related genes. **C)** Information of predicted interaction between miR-141/miR-200a and *EGR2* obtained from miRDB, microT-CDS and TargetScan databases. **D)** Potential binding sequences of miR-141 and miR-200a within the 3′UTR of EGR2 retrieved from TargetScan database.

to Venn diagrams for each miRNA (**Supplementary Excel files 1 and 2, sheet 1**). This strategy resulted in fewer but more confident target genes which are also correlated with Th17 cell lineage. Interestingly, us *in-silico* target prediction analysis identified *RHEB*, *RARB* and *EGR2* as common target genes for both miRNAs (**Fig. 3A, 3B**). Finally, by extracting experimentally validated miRNA-target pairs from TarBase and miRTarBase, *EGR2* was selected as the candidate target gene of miR-141/miR-200a in the Th17 cell development process and used in our subsequent analyses. The specific characteristics of possible interaction between miR-141/miR-200a and EGR2-3′UTR and their predicted binding sequences have been shown in **Figure 3C, 3D**.

4.6. Gene-Based Interaction Network Described a Set of Significant Interactions Between EGR2 and Autoimmunity and Th17 Cell Genes

In order to verify the *EGR2* correlation with Th17 cell development and autoimmune conditions and identify the possible deregulated gene interactions following miR-141 and miR-200a abnormal upregulation, we constructed a gene-based interaction network. This interaction network consisted of 115 Th17-associated genes and 55 autoimmunity-related genes and was analyzed using the STRING database (**Supplementary Excel file 4**). The Interaction Score > 0.7 was set as the cutoff value and then significant interactions were visualized via Cytoscape software (**Fig. 4**). Our gene-based interaction network analysis exhibited important interactions between *EGR2* and *EGR1* (from autoimmunity genes) and *RARA*, *RARG*, *EP300* and *BATF* (from Th17 cell genes) that may be differentially regulated after *EGR2* targeting by miR-141/miR-200a.

4.7. Molecular Signaling Pathway Enrichment Analysis of EGR2 Co-Expressed Genes Specified their Pivotal Roles in the Immune Response Homeostasis

For further evaluating the significance of *EGR2* in Th17 cell restriction and autoimmunity inhibition, we performed a pathway enrichment analysis for the genes mostly co-expressed with *EGR2* using Enrichr (Supplementary Excel file 5). In this context, statistically

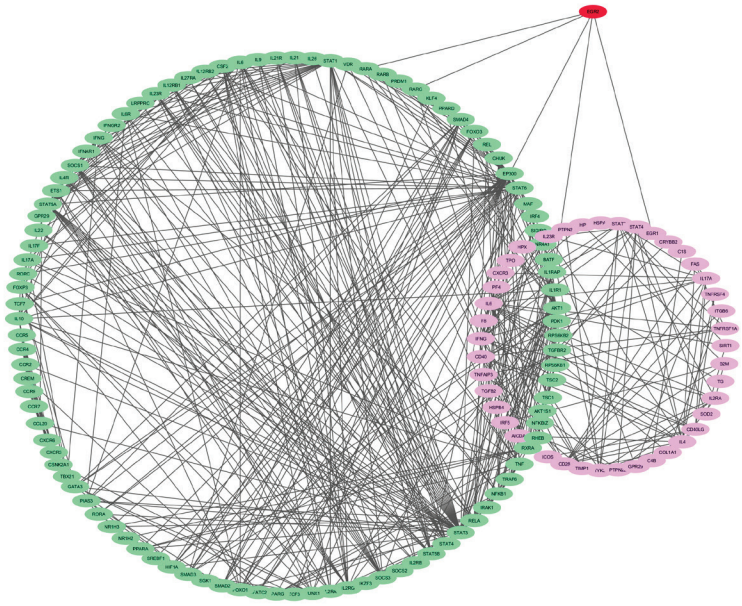


Figure 4. Gene-based inteaction network. Significant interactions (Interaction Score > 0.7) of *EGR2* with genes correlated with Th17 cell differentiation or autoimmune diseases were analyzed using STRING database and visulaized by Cytoscape software. The green nodes indicate Th17 cell-related genes and the pink nodes present autoimmune diseases-associated genes.

meaningful KEGG, PANTHER and REACTOME signaling pathways (p<0.05) were summarized and visualized via Appyters (**Fig. 5A, 5B, 5C**). The results of our pathway enrichment analysis indicated that *EGR2* co-expressed genes were part of signaling pathways correlated with immune response homeostasis including Toll-like receptor signaling pathway, IL-17 signaling pathway, inflammation mediated by cytokine signaling pathway, T cell activation, B cell activation, cytokine signaling in the immune system and innate immune system. Notably, these *in-silico* findings showed that *EGR2* and its co-expressed genes are also implicated in fundamental signaling pathways of Th17 cell development which can be improperly altered upon *EGR2* deregulation (**Supplementary Figs. 1, 2**).

4.8. Th17 Cell Differentiation

To establish an *ex-vivo* Th17 cell differentiation system, isolated naïve T cells were cultured under a Th17 cell polarizing medium for six days (**Supplementary Fig. 3**). Our morphological analysis indicated that within the 48 h of Th17 cell induction, cell clumps which are the main morphological characteristic of Th17 cell differentiation appeared in all cultured wells (**Fig. 6A**). To confirm the morphological evidence of Th17 cell skewing, we assessed IL-17 concentration on days 0, 2, 4 and 6 of the differentiation period by means of ELISA. As illustrated in **Figure 6B**, IL-17 secretion increased remarkably from the second day and reached a maximum of 701pg.mL^{-1} on the sixth day post Th17 cell induction (p=0.01). Additionally, q-PCR analyses demonstrated that hallmark genes of Th17 lineage upregulated significantly from day 2 of differentiation period. To be precise, the expression level of *RORC* and *IL-23R* reached, respectively, 5.3 (p=0.042) and 6.03 (p=0.005) folds higher than their expression on day 0 (**Fig. 6C, 6D**). These findings verified the successful establishment of Th17 cell induction process.

4.9. PBX1 is Upregulated During the Th17 Cell Differentiation Process

To clarify whether PBX1 is a positive regulator of Th17 cell development, we first investigated its expression in the thymus, spleen and lymph nodes since it is a prerequisite to be involved in T helper cell differentiation.

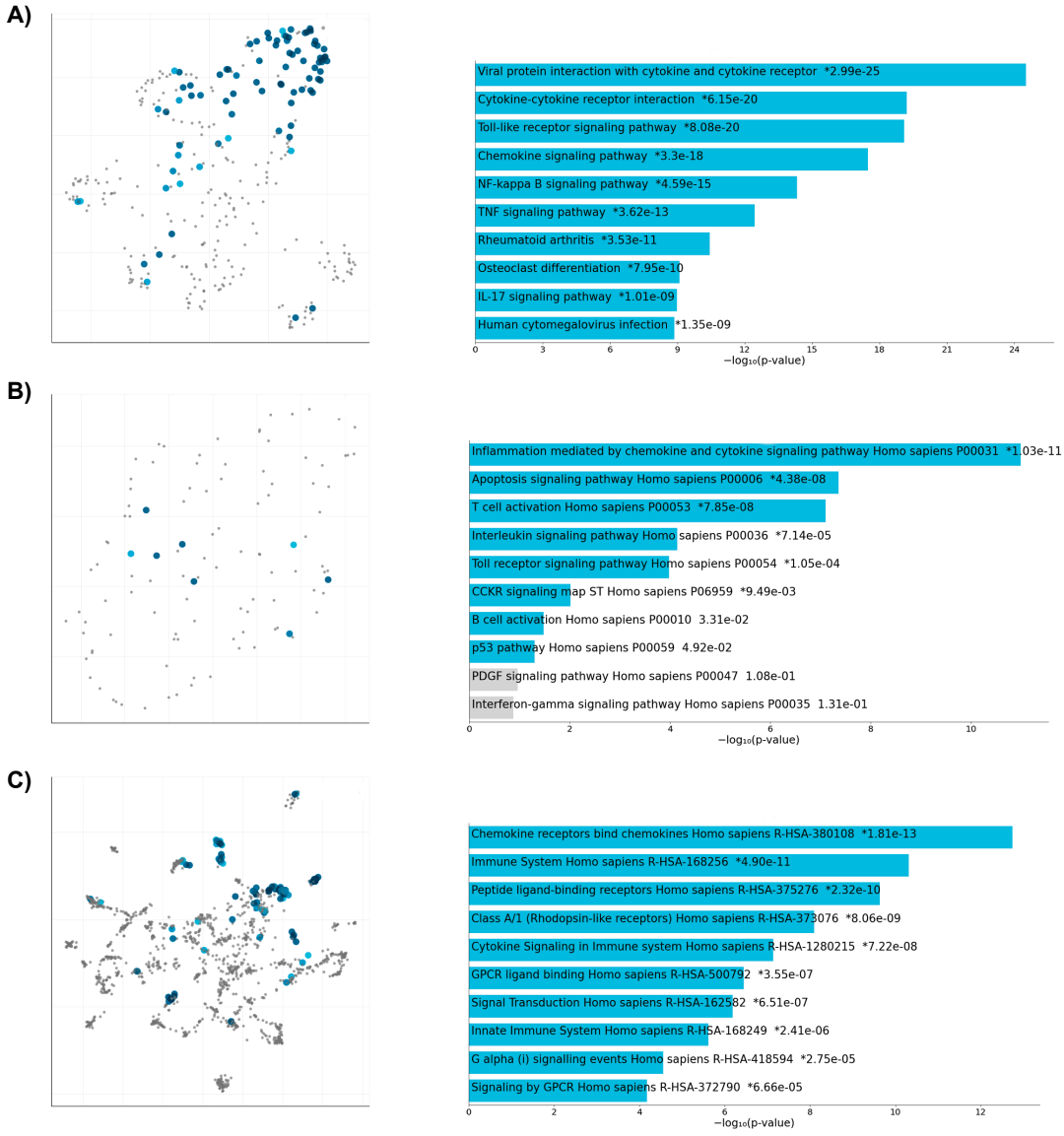


Figure 5. Molecular signaling pathway enrichment analysis. (A, B, C) Top statistically related signaling pathways to *EGR2* co-expressed genes obtained from KEGG, PANTHER and REACTOME databases by means of Enrichr tool. Scatter plots are organized so that similar gene sets form cluster together. The larger blue points indicate significantly enriched terms- the darker blue, the more meaningful term and the smaller *p* value. The gray points are not significant. Bar charts show the top 10 enriched signaling pathways along with their corresponding *p* value. Colored bars indicate significant pathways ($p < 0.05$). A (*) next to the *p* value shows that the signaling pathway also has a significant adjusted *p* value (<0.05).

Analyzing the FANTOM5 dataset retrieved from The Human Protein Atlas confirmed the protein expression of PBX1 in the mentioned organs (**Supplementary Fig. 4**). Next, we quantified the expression levels of *PBX1* gene during the Th17 cell differentiation period. As shown in **Figure 6E**, *PBX1* exhibited a constant increasing expression pattern over the induction process as its transcript levels reached the highest amount on day 6 by a mean factor of 3.93 (p=00.24). These results, taken together with us *in-silico* predictions, indicate that increased expression of *PBX1* may positively affect the Th17 cell development through upregulation of miR-141 and miR-200a.

4.10. EGR2 is a Direct Target of MiR-141 and MiR-200a

Based on our aforementioned bioinformatic prediction, we hypothesized that *EGR2* is a common target gene of miR-141 and miR-200a in Th17 cell differentiation process. We designed dual-luciferase reporter assays to validate this assumption and verify the relationship between *EGR2* and miR-141/miR-200a. Our results demonstrated that luciferase activity of EGR2-WT decreased, while that of EGR2-MUT showed no changes in HEK293T cells following transfection of p-Bud-EGFP vectors containing either miR-141 (p=0.0001) or miR-200a (p=0.0007) precursors (**Fig. 6F, 6G**). Additionally, enzyme activity was not affected in the transfected cells with negative control p-Bud-EGFP. In support, the results of q-PCR shown in Fig. 6H indicated a diminished gene expression of *EGR2* after day 2 of differentiation in the way that its expression level on day 6 dropped more than 75 percent compared to day 0. These data reflected that miR-141 and miR-200a can target *EGR2* gene during the Th17 cell differentiation and inhibit its expression.

4.11. SOCS3 is Downregulated During the Th17 Cell Differentiation Process

SOCS3 is known to be upregulated upon EGR2 binding to its regulatory sequence and this interaction is a critical step in limiting Th17 cell differentiation. Therefore, we speculated that *SOCS3* expression should be declined after miR-141/miR-200a-driven *EGR2* gene silencing over the Th17 cell development. Finally, qRT-PCR examination of *SOCS3* expression level was performed, showing a downward trend exactly similar to that of *EGR2* (**Fig. 6I**). The transcriptional level of *SOCS3* reduced approximately by 60 percent from day 2 of differentiation to day 6 (p=0.01). These findings demonstrated that *EGR2* inhibition by miR-141 and miR-200a may subsequently lead to reduced expression of *SOCS3*.

5. Discussion

Th17 cells play a dual role in the host defense and autoimmune pathogenesis (21). However, how the switch from protection to harm occurs during autoimmune disorders still needs to be investigated. Therefore, focusing on deregulated molecular networks involved in excessive differentiation of Th17 cells may provide new perceptions into the possible underlying mechanisms of Th17-mediated autoimmunity which can be used in future studies on the novel therapeutic targets for autoimmune disorders.

In this study, we aimed to investigate a potential molecular mechanism active in the development of Th17 cells and found that the PBX1 transcription factor is implicated in Th17 cell induction probably through the upregulation of miR-141 and miR-200a. Furthermore, we demonstrated that *EGR2* is a downstream direct target of these two miRNAs and its miR-141/miR-200a-driven inhibition during Th17 cell differentiation may lead to downregulation of another principal negative regulator of Th17 cell skewing, *SOCS3*. These findings not only point out the roles of *PBX1*, *EGR2* and *SOCS3* signals in miR-141/miR-200a-mediated Th17 cell differentiation but also point to a possible deregulated PBX1/miR-141-miR-200a/EGR2/SOCS3 axis involved in Th17–triggered autoimmunity.

Our previous data demonstrated that improper overexpression of miR-141 and miR-200a is associated with Th17 cell differentiation (16). In the current study, we further explore the reason for the upregulated expression of miR-141 and miR-200a during the Th17 cell induction process. Using a series of bioinformatic predictions performed via the TransmiR database and through a subsequent common-screening strategy, we identified PBX1, Egr1 and Tcf3 as common TFs regulating miR-141 and miR-200a. The direct interaction of these TFs with the regulatory elements of miR-141 and miR-200a has not been reported yet in literature and the TransmiR database predicted them based on ChIP-seq data, TF binding motifs and transcriptome profiles. Since PBX1 is the only predicted TF that contributes positively to autoimmunity, we selected it as the potential TF involved in miR-141/miR-200a overexpression (13, 22, 23). Next, our computational DNA sequence analysis applying JASPAR, rVista and hTFtarget databases predicted several PBX1 binding motifs on miR-141 and miR-200a promoter sequences and therefore further confirmed the potential interaction between PBX1 and miR-141/miR-200a regulatory elements. Going further, using an *ex-vivo* human Th17 cell differentiation system and qPCR assays, we verified increased transcription of *PBX1* over the induction period and, hence, confirmed its association with Th17 cell differentiation for the first time. PBX1 is a homeodomain-containing TF that regulates chromatin accessibility to various genes (24).

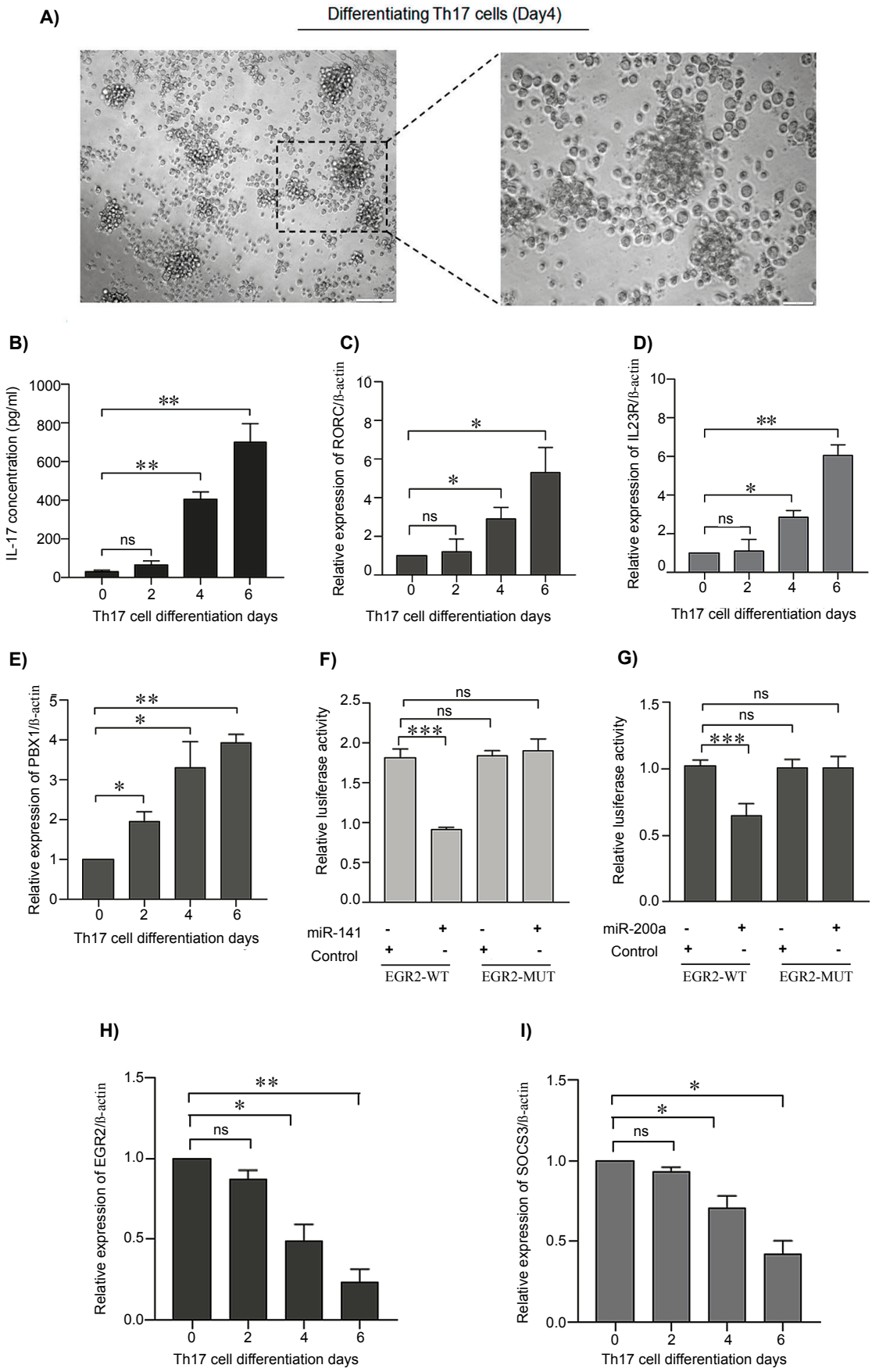


Figure 6. Legend continued on next page.

In the immune system, a previous study corroborated that the expression of retinoic acid (RA) is under the transcription control of Pbx TFs (25). Since RA plays a pivotal role in the development of Treg cells (a T cell subpopulation that acts to suppress autoimmune responses), more recent studies explored the function of PBX1 in Treg cell differentiation and autoimmune conditions (26). For instance, Choi et al. showed the overexpression of a variant of *PBX1* in $CD4^+$ T cells of lupus patients resulting in impaired development of Treg cells and increased numbers of autoreactive Tfh cells (13). Additionally, two other studies have provided evidence for increased levels of phosphorylated STAT3 protein upon transfection of *PBX1* expression plasmids in HEK cells (27, 28). These previous results in conjunction with our computational predictions and experimental data support a positive relationship exists between PBX1 and Th17 cell differentiation probably through the upregulation of miR-141 and miR-200a.

Another focus of the current study was the possible miR-141/miR-200a-driven regulatory mechanism in Th17 cell differentiation. Imperfect miRNA-mRNA interaction is common and therefore it has been widely reported that one miRNA can regulate multiple genes as each gene can be targeted by multiple miRNAs (29, 30). In our previous paper, we reported that miR-141 and miR-200a can be involved in Th17 cell differentiation by targeting *retinoic acid receptor beta* (*RARB*) (16). Herein, we explored other possible deregulated gene expressions induced by miR-141/miR-200a abnormal overexpression to obtain a more comprehensive view of disordered interaction networks implicated in Th17 cell skewing. Using a consensus-based *in-silico* prediction approach and considering strict selection thresholds, we identified *EGR2* as the other potential target of miR-141 and miR-200a in the pathway of Th17 cell development. Although the analysis of online prediction data is a widely used strategy in the context of miRNA-target pair identification, laboratory examinations besides the prediction results can lead to heighten the accuracy of predictions. In the present study, in line with us *in-silico* prediction, our qPCR and luciferase reporter assay results confirmed the direct interaction between miR-141/miR-200a and 3´UTR of EGR2 mRNA. Interestingly, *EGR2* is known to be a downstream target of RA signaling and RARß function (31). The significance of this phenomenon is reflected by the aforementioned fact that RA is an amplifier of Treg cells and a negative controller of Th17 lineage (26). Therefore, our current findings are perfectly consistent with our previous data, suggesting a bidirectional role of miR-141 and miR-200a in RA signaling inhibition and therefore in Th17 cell development through targeting *RARB* and *EGR2*. Taken together, the results of this part of the study have illustrated that miR-141 and miR-200a inhibit the expression of *EGR2*, which is another indispensable negative regulator of Th17 cells, further highlighting the importance of elevated expression of these two miRNAs in Th17 cell polarization.

Aiming at exploring the possible dysregulated gene interactions and interrupted signaling pathways induced by *EGR2* silencing, we performed gene interaction network and pathway enrichment analyses, respectively. Our gene interaction analysis revealed significant interactions of *EGR2* with *RARA*, *RARG*, *BATF*, *EP300* and *EGR1* genes, all of which are strictly associated with Th17 cell generation or autoimmune conditions. As mentioned above, EGR2 functions downstream of RA

Figure 6. (A to D) Human Th17 cell differentiation. A) Bright field images of differentiating Th17 cell on day 4 of induction period with different magnification (20x and 40x). Large cell clumps are the main characteristic of Th17 cell differentiation in *ex-vivo* cultures. **B)** Protein levels of IL-17 secreted by differentiating cells on days 0, 2, 4 and 6 of induction examined by ELISA (** $p < 0.01$, students t-test). **(C, D)** Expression monitoring of *RORC* and *IL-23R* as master markers of Th17 lineage using qRT-PCR. Gene expressions were normalized to *ß-actin* levels as internal reference (* $p < 0.05$, ** $p < 0.01$, students t-test). ns stands for non-significance. (E to I) Upstream regulation and downstream targeting of miR-141 and miR-200a. **E)** qPCR analysis of *PBX1* as upstream transcription factor regulating miR-141/miR-200a expression. **(F, G)** Binding analysis of miR-141 and miR-200a to the 3´UTR of EGR2 mRNA using dual luciferase reporter gene assays. **(H, I)** Transcript levels of *EGR2* and *SOCS3* over the Th17 cell differentiation period measured by qPCR. Gene expression results are normalized to expression levels of the reference gene, *ß-actin*. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$, student t-test). ns stands for non-significance.

signaling pathway that embark upon RA binding to its nuclear receptors including RARα, RARß and RARγ ,respectively encoded by *RARA*, *RARB* and *RARG* genes (32, 33). Regarding the role of Egr1 in Th17 cell development and autoimmune pathology, it has been documented that this transcription factor serves as a gatekeeper of inflammatory factors and maintains Treg/Th17 balance (34). In contrast to the inhibitory role of RA and Egr1 in Th17 cell polarization, Batf promotes and stabilizes Th17 differentiation (35). Although EGR2 does not regulate Batf expression, it has been reported that its binding to Batf blocks the association of this protein with regulatory sequences of *IL-17* gene and, hence, inhibits Th17 cell induction (36). *EP300* is another essential requisite for Th17 cell polarization and the hypothetical interaction between *EGR2* and *EP300* predicted by our gene interaction analysis may explain the other aberrant gene interaction caused by higher levels of miR-141 and miR-200a (37). Collectively, it can be inferred from our gene-based interaction network findings that by targeting the *EGR2,* miR-141 and miR-200a are likely to interfere in key gene interactions crucial for Th17 cell limiting and autoimmunity prevention. However, more laboratory tests would be needed to verify the predicted interactions. Besides, the significantly obtained signaling pathways of our pathway enrichment analysis further emphasized the significance of *EGR2* and its co-expressed genes in pathways already linked to immune system homeostasis and demonstrated that reduced expression of *EGR2* can cause detrimental effects on the signaling pathways delicately regulating immune responses.

Accumulating evidence has reported that EGR2 limits the proliferation and cytokine production of Th17 cells by positively regulating *SOCS3*(38). *SOCS3* is a chief suppressor of STAT3 activation and therefore regulates STAT3-mediated IL-17 secretion and Th17 cell differentiation (39). In support, *SOCS3*-deficient T cells have been reported to be more susceptible to Th17 cell development (40). In line with these findings, our qPCR analysis exhibited a significant reduction in *SOCS3* expression over the Th17 differentiation period. Considering the aforementioned data that PBX1 improves STAT3 activation, the results of this part of our study further confirm the positive correlation between PBX1 and Th17 cell differentiation probably through miR-141/miR-200a upregulation and subsequent *EGR2* and *SOCS3* inhibition. However, stronger laboratory evidence is required to prove this.

6. Conclusions

In conclusion, the findings of the current study demonstrated that *PBX1* is involved in Th17 cell differentiation probably via miR-141/200a-mediated *EGR2* and *SOCS3* inhibition. Thus PBX1/miR-141-200a/EGR2/*SOCS3* signaling axis may serve as a novel player in Th17 cell-driven autoimmunity. In addition, us *in-silico* gene interaction and pathway enrichment analyses discover connection between *EGR2* expression pattern during Th17 cell differentiation and higher-level information on possible subsequent affected gene interactions and signaling pathways, thereby elucidating the other potential biochemical mechanisms underlying miR-141/200a-driven Th17 cell polarization. However, more *in-vitro* and *in-vivo* experiments are essential to further confirm the findings and predictions of this study and evaluate the possible translational potential of PBX1/mir-141-miR-200a/EGR2/*SOCS3* axis in autoimmune disorders.

Ethics approval and consent to participate

The use of human samples in this study was approved by the ethics committee of Royaninstitute (code No. 378IR.ACECR.ROYAN.REC.1396.111).

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This project was financially supported by the National Institute for Medical Research Development (NIMAD's project no. 942792).

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