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Original Article

microRNA and mRNA interactions in induced pluripotent stem cell reprogramming of lymphoblastoid cell lines

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Abstract: A large number of Epstein Barr virus (EBV) immortalized lymphoblastoid cell lines (LCLs) have been generated and maintained in genetic/epidemiological studies as a perpetual source of DNA and as a surrogate *in vitro* cell model. Recent successes in reprograming LCLs into induced pluripotent stem cells (iPSCs) has paved the way to generate more relevant *in vitro* disease models using this existing bioresource. However, the latent EBV infection in the LCLs make them a unique cell type by altering expression of many cellular genes and miRNAs. These EBV induced changes in the LCL miRNome and transcriptome are reversed upon reprogramming into iPSCs, which allows a unique opportunity to better understand the miRNA and mRNA interactions that are EBV induced in LCLs and the changes that takes place during iPSC reprogramming. To identify the potential miRNA-mRNA interactions and better understand their role in regulating the cellular transitions in LCLs and their reprogrammed iPSCs, we performed a parallel genome-wide miRNA and mRNA expression analysis in six LCLs and their reprogrammed iPSCs. A total of 85 miRNAs and 5,228 mRNAs were significantly differentially expressed (DE). The target prediction of the DE miRNAs using TargetScan-Human, TarBase and miRecords databases identified 1,842 mRNA targets that were DE between LCLs and their reprogrammed iPSCs. The functional annotation, upstream regulator and gene expression analysis of the predicted DE mRNA targets suggest the role of DE miRNAs in regulating EBV induced changes in LCLs and self-renewal, pluripotency and differentiation in iPSCs.

Keywords: Human, lymphoblastoid cell line, iPSC reprogramming, miRNA, mRNA, gene expression regulation

Introduction

Induced pluripotent stem cells (iPSCs) reprogrammed from cryopreserved lymphoblastoid cell lines (LCLs), represent a uniquely valuable resource for biomedical research in disease modeling, disease gene identification and for screening new therapeutics, because LCLs collected in genetic and epidemiological studies represents one of the largest, well-characterized, existing bioresources available for iPSC reprogramming. Furthermore, a multitude of phenotypic , genotypic and omics data already exists on their donors [1].

Over the last few years, the reprogramming methodology of cryopreserved LCLs has improved significantly [2]. In our own work, we have a 100% success rate and a very high reprogramming efficiency in reprogramming cryopreserved LCLs from more than 150 participants of our San Antonio Family Heart Study (SAFHS). We have also published a detailed account of miRNAs and mRNAs that are differentially expressed during iPSC reprogramming of cryopreserved LCLs [2].

However, the latent Epstein Barr virus (EBV) infection in the LCLs make them a unique cell type compared to a more conventionally used dermal fibroblast or other primary cells. In LCLs, EBV typically persists as non-integrated episomal plasmids and establishes type III latency program characterized by the expression of a subset of viral genes i.e. six EBV nuclear antigens (EBNA-1, EBNA-2, EBNA-3A/B/C, EBNA-LP), three latent membrane proteins (LMP-1, LMP-2A/B), and non-coding RNAs (EBER1/2,

BART) including some viral microRNAs (miRNAs) are expressed [3]. The latently expressed EBV oncoproteins exploit the intrinsic transcription programs of B-lymphocytes to achieve immortal cell growth resulting in the altered expression of a number of cellular genes and miRNAs [4-6]. Therefore, LCLs have been exploited as an *in vitro* cell model for EBV-associated lymphoproliferative diseases [7].

We have previously shown that the EBV induced changes in the cellular transcriptome of both mRNA and miRNA expression were reversed upon iPSC reprogramming of the LCLs [2]. Furthermore, the down regulation of these EBV induced transcriptomic changes suggest significant inhibition of EBV genome transcriptional activity in the reprogrammed iPSCs, which is essential for the EBV genome replication in the host cell, therefore iPSC reprogramming results in a gradual removal of the EBV genomes from reprogrammed iPSCs [8, 9].

These transcriptomic events that occur during the iPSC reprogramming of LCLs allows a unique opportunity to better understand the transcriptomic changes that are EBV induced in LCLs and the changes that takes place during iPSC reprogramming including miRNA and mRNA interactions. In this manuscript we have analyzed our existing mRNA and miRNA sequence data, generated from six LCLs and their reprogrammed iPSCs, to investigate the miRNA - mRNA interactions during these cellular transitions.

Material and methods

A detailed description of the methodology used in iPSC reprogramming of the six cryopreserved LCLs, iPSC characterization, validation and RNA sequencing of the LCLs and their reprogrammed iPSCs can be found in our previous publication [2]. Briefly, the 6 de-identified LCLs established from our San Antonio Family Heart Study (SAFHS) participants from whom appropriate written consent was obtained were reprogrammed using our highly efficient feeder free episomal plasmid based methodology [2]. The reprogrammed iPSC lines formed flat and compacted colonies and showed high nucleus to cytoplasm ratios. The immunocytochemistry (ICC) and differential gene expression analysis showed that all of our reprogrammed iPSC lines expressed pluripotency markers and showed potential to differentiate into cells of all three germ layers (**Figure 1**). The six LCLs and their well characterized and validated iPSC lines were RNA sequenced using Illumina TruSeq Small RNA and TruSeq mRNA sample preparation kits on the Illumina HiSeq 2500 next generation sequencing platform. Institutional Review Board and Institutional Biosafety Committee of the University of Texas Rio Grande Valley (Edinburg, TX) approved all protocols used in this study.

Sequence analysis

Raw fastq sequence files were generated and demultiplexed using the Illumina CASAVA v1.8 pipeline. After pre-alignment QCs, sequences were aligned to human genome build 19 (hg19) and mapped to RefSeq transcripts using StrandNGS software v3.3 (Strand Genomics Inc.). Small RNA reads were mapped to small RNA annotations inferred from miRbase (v20), ensemble (e75), gtrnadb (tRNA) and UCSC knowGene tables (piRNA) and implemented in Strand NGS software. The aligned reads were filtered based on default read quality matrix and log transformation and "DESeq" normalization was applied. The known miRNAs and mRNAs having a normalized read count (NRC) ≥ *20* in all 6 LCLs or their reprogrammed iPSCs or in both were considered expressed and selected for differential gene expression analysis.

Differential gene expression analysis

To identify differentially expressed (DE) miRNAs and mRNAs, moderated *t* statistics and expression fold change analyses ware performed on miRNA and mRNA data sets. The miRNAs and mRNAs having moderated *t* statistic *FDR corrected p-value* ≤ *0.05* and *FC absolute* (*FC-abs*) ≥ *2.0*, were considered DE.

miRNA target prediction

The miRNA target filter implemented in Ingenuity Pathway Analysis (IPA) platform, which uses the TargetScan-Human, TarBase and miRecords databases, was used to identify predicted and experimentally observed miRNA targets. TargetScan content in IPA uses TargetScan algorithm predicted mRNA targets of human miRNAs that are binned into high and moderate confidence. TarBase content identifies experimentally demonstrated miRNA-mRNA interac-

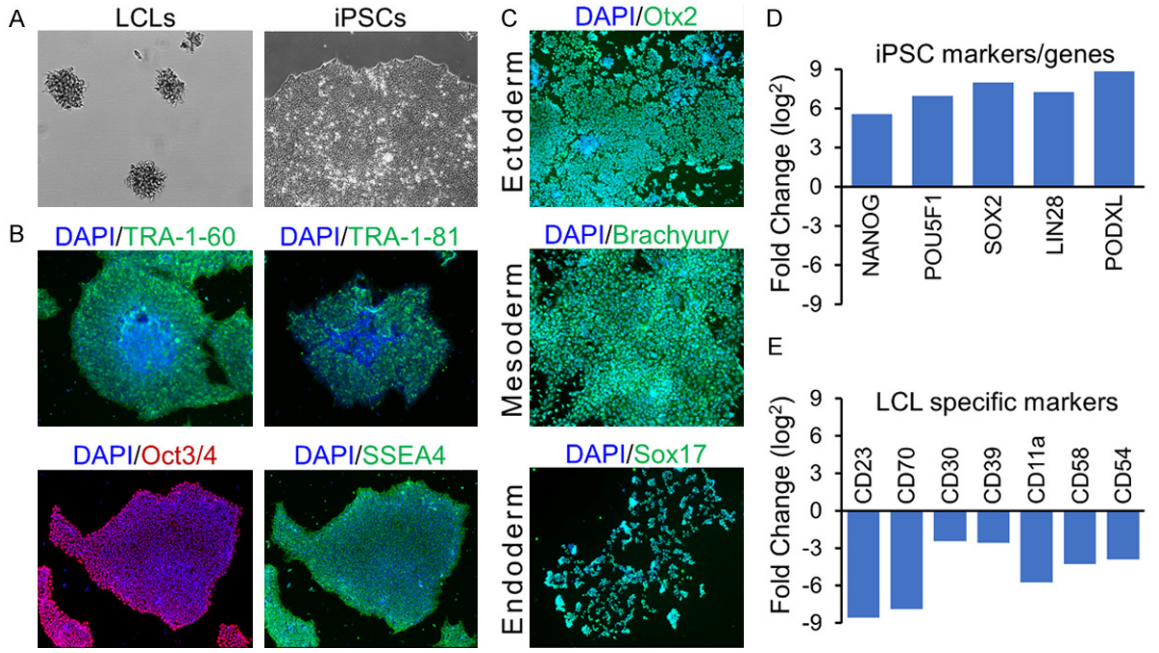

Figure 1. Validation of the LCL reprogrammed iPSC lines. (A) Bright filed image of LCLs and reprogrammed iPSCs, (B) ICC analysis of the generated iPSCs showing expression of the pluripotency markers, (C) ICC analysis of the 3 embryonic germ layers differentiated from the reprogrammed iPSCs, (D, E) graphs showing differential gene expression of iPSC core pluripotency markers/genes and LCL specific markers.

tions from TarBase using miRbase identifiers. And miRecords content uses experimentally validated human, rat and mouse miRNA-mRNA interactions from published literature. The TargetScan-Human predicted mRNA targets binned into high confidence and experimentally observed mRNA targets from TarBase and miRecords that were significantly DE between LCLs and their reprogrammed iPSCs, were considered for functional analysis.

Functional annotation and gene expression analysis

Functional annotations, upstream regulator analysis (URA) and pathway analysis were performed on identified mRNA target gene expression data sets using IPA. Right-tailed Fisher's exact test *p* values corrected for FDR were used to calculate enrichment significance. The direction of functional change in URA was assessed by activation Z-score as implemented in IPA. Further details of these methods implemented in IPA can be found in [10].

Results and discussion

The smallRNA and mRNA sequence data generated from 6 LCLs and their validated iPSC lines (**Figure 1**) was submitted to gene expression omnibus (GEO) archive (accession number GSE74289). A total of 5.5 and 8.3 million small RNA 40 bp single-end reads and 28.4 and 29.9 million mRNA 100 bp paired-end reads were obtained for LCLs and their iPSCs, respectively.

Differentially expressed genes

To identify miRNA and mRNA interactions that are EBV induced in LCLs and the changes that take place during iPSC reprogramming, it was important to first identify the miRNAs and mRNAs that were significantly DE during the iPSC reprogramming of LCLs. As stated in the materials and methods section, only known miRNAs and mRNAs having NRC ≥ *20* in all 6 samples of one or both cell types were considered expressed for differential gene expression analysis. The moderated *t*-statistics and expression fold change (FC) analysis following the criteria, *FDR corrected p-value* ≤ *0.05* and *FC-abs* ≥ *2.0*, identified 85 miRNAs and 5,228 mRNAs that were significantly DE between LCLs and their reprogramed iPSCs (**Figure 2A**, **2B**; Supplementary Table 1).

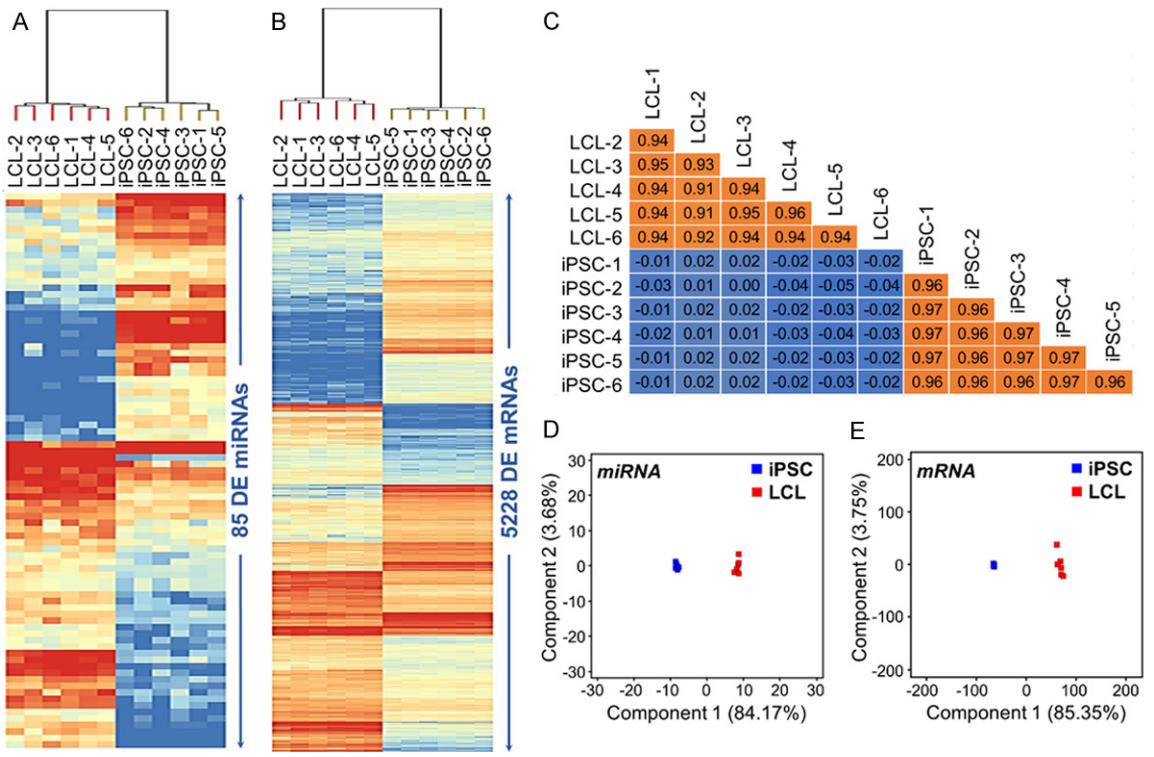

Figure 2. miRNA and mRNA differential expression analysis between LCLs and their reprogrammed iPSCs. (A) heat map of 85 DE miRNAs, (B) heat map of 5,228 DE mRNAs, (C) correlation coefficient (r^2) plots calculated for DE miRNA and mRNA between LCLs and their reprogrammed iPSCs, (D, E) Principal component analysis (PCA) based on DE miRNAs and mRNAs found DE between LCLs and their reprogrammed iPSCs.

The expression heat maps, correlation analysis (*correlation coefficient = 0.94 ± 0.006 at 95% CI for LCLs and 0.96 ± 0.002 at 95% CI for iPSCs*) and the principal component analysis of the DE miRNAs and mRNAs in six LCLs and their reprogrammed iPSCs (**Figure 2A-E**) suggest discrete and uniform resetting of the miRNome and transcriptome during iPSC reprogramming.

miRNAs expressed in LCLs and their reprogrammed iPSCs: The most abundant cellular miRNAs expressed in LCLs and showing significant down regulation in reprogrammed iPSCs are shown in **Figure 3A**. Together, these 46 miRNAs accounted for ~70% of the expressed LCLs miRNome (**Figure 3C**) and were highly enriched in miRNAs (*miR-155, let-7a-i, miR-146a/b, mir-29a, miR-142, miR-181*, miR-150, miR-10a)* that have been reported to be upregulated by EBV infection in LCLs [4, 11-13]. A total of 39 miRNAs were significantly upregulated in reprogrammed iPSCs (**Figure 3B**) and these miRNAs together accounted for ~65% of the reprogrammed iPSCs miRNome (**Figure 3C**). The miRNAs associated with the human iPSC/ESC pluripotency and maintenance (*miR-302a/b/c/d, miR-371/372/373, miR204, miR-92b*) were the most upregulated miRNA clusters/families [14, 15].

mRNAs expressed in LCLs and their reprogrammed iPSCs: A total of 2,317 mRNAs, which together accounted for ~32% of the LCLs expressed transcriptome, were significantly down regulated in LCL reprogramed iPSCs (**Figure 3D**). The mRNAs encoding B-cell transcription factors, major histocompatibility complex classes I and II, cell surface markers and cellular adhesion molecules that are known to be expressed in EBV latency III infection in LCLs were highly enriched in these down regulated mRNAs. A total of 2,911 mRNAs were significantly upregulated in the reprogrammed iPSCs and they accounted for ~30% of the iPSCs expressed transcriptome (**Figure 3D**). The mRNAs/genes that are known to be involved in stemness, pluripotency and self-renewal of human iPSCs were highly enriched in the upregulated mRNAs. A detailed description of LCL

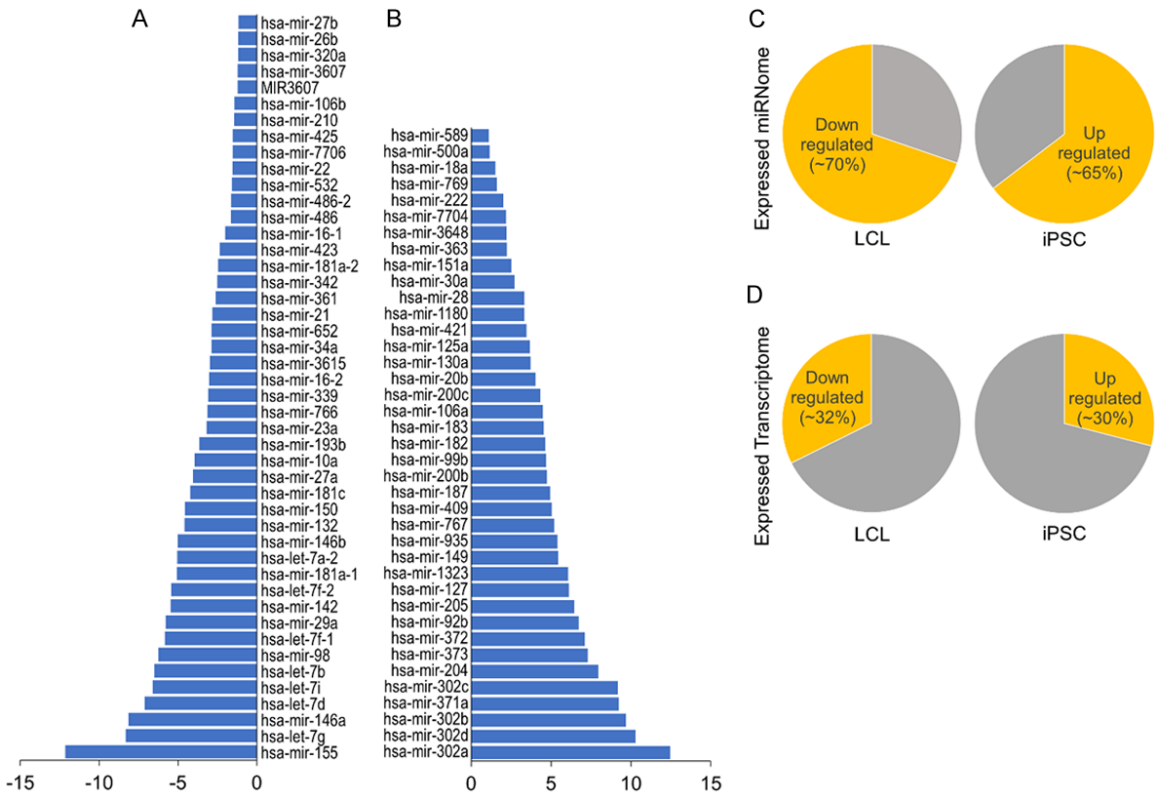

Figure 3. Differentially expressed miRNA and proportion estimates of DE miRNome and DE transcriptome in LCLs and iPSCs. (A) bar graph of significantly down regulated miRNAs, (B) bar graph of significantly up regulated miRNAs, (C) pie graph showing proportion of DE vs total expressed miRNome in LCLs and reprogrammed iPSCs, (D) pie graph showing the proportion of DE vs total expressed transcriptome in LCLs and reprogrammed iPSCs.

and iPSCs DE transcriptome can be found in our previous publication [2].

In-silico miRNA target prediction and their interaction with LCL-iPSC DE transcriptome

Having established the miRNAs and mRNAs that were upregulated by the EBV latency III infection in LCLs and showed significant down-regulation in reprogrammed iPSCs and the miRNA and mRNAs that were significantly upregulated in the reprogrammed iPSCs, we next sought to determine the mRNA targets of these DE miRNAs. First, we implemented computational miRNA target prediction using TargetScan-Human, TarBase and miRecords data bases in IPA. The 85 DE miRNAs mapped to 67 mature miRNA families and 52 of these miRNA families had targeting information available. Following the criteria that the interaction sites were either experimentally observed and/or predicted with high confidence, 6,008 target mRNAs resulting into 8,215 total miRNA-mRNA interactions were identified. 1,842 of these mRNA targets were DE between LCLs and their reprogrammed iPSCs and resulted in 2,642 miRNA-mRNA interactions (Supplementary Table 2), 1,407 of these predicted interactions were between miRNA-mRNA pairs that were oppositely expressed. Since DE genes are regulated by diverse genetic and epigenetic mechanisms during iPSC reprogramming of LCLs, we have included all DE mRNAs in the miRNA target predictions in further analysis irrespective of the directions of miRNA-mRNA expressions.

LCL specific miRNA-mRNA interactions: The 27 miRNA families that were expressed in LCLs but significantly down-regulated upon reprogramming, were predicted to target 1,141 mRNAs that were DE between LCLs and their reprogrammed iPSCs (resulting in 1,425 miRNA-mRNA interactions). Of these miRNA-mRNA interactions, 244 were experimentally observed in other studies (Supplementary Table 2). To further validate our findings, we queried our unique mRNA targets identified by LCL specific miRNA-mRNA interactions against high confi-

dence 3' UTR miRNA-interaction sites identified by Argonaute 2 (Ago-2) based PER-CLIP analysis of LCLs in a previously published study Skalsky et al., [4]. Of the 1,141 mRNA targets, 324 (~29%) were also among the mRNA targets identified by Ago-2 based PER-CLIP analysis of the LCLs, suggesting a high concordance between the two studies. Next, we focused on *miR-155,* the most abundant cellular miRNA in LCLs. The EBV encoded EBNA2, EBNALP and LMP1-acivated NF-κB subunit upregulates *miR-155* in LCLs and its expression is essential for LCL survival in *in vitro* cultures [6, 16]. Expression of *miR-155* is implicated in a number of B-cell malignancies and plays an important oncogenic role in herpesvirus biology as both Kaposi's sarcoma-associated herpesvirus (KSHV) and Marek's disease virus (MDV) encode functional *miR-155* analogs [4]. We have identified 77 DE mRNAs which were the predicted targets of *miR-155* in our data set and 55 of these targets were experimentally observed in previous studies in IPA (Supplementary Table 2). The functional annotation and gene expression analysis of identified *miR-155* targets (77 DE mRNAs) showed very high enrichment in Cancer (74 molecules; *p-value range 1.88E-03 - 1.48E-08*) and in cellular growth and proliferation functional categories (36 molecules; *p-value range 1.81E-03 - 7.01E-09*). An upstream regulator analysis of the *miR-155* targets, predicted activation of *SOX2, SYVN1* and *ESR1* (activation *z-score* ≥ *2.0*) regulated genes and inhibition of *TAL1, TNFSF11* and *TP53* (activation *z-score* ≤ *-2.0*) regulated genes, which suggest that *miR-155* down regulation promotes LCL to iPSC reprogramming.

miRNA-mRNA interactions of miRNAs upregulated in iPSCs: The 25 miRNA families which were significantly upregulated in reprogrammed iPSCs, were predicted to target 1,002 DE mRNAs and resulted in 1,217 total miRNA-mRNA interactions. About 14% of these interactions were experimentally observed in previous studies (Supplementary Table 2). The human embryonic stem cell (ESCs) and iPSCs express a unique set of miRNAs with the majority transcribed from two genomic loci, the *miR-302* cluster encoding *miR-302a/b/c/d* and *miR-371-373* cluster encoding *miR-371, miR-372* and *miR-373* [17, 18]. The miR-302 and miR-371-373 clusters and a highly related miRNA, *miR-92b* accounted for the majority (~67%) of the DE miRNome in our reprogrammed iPSCs and were predicted to target 143 DE mRNAs. The functional annotation and gene expression analysis of these 143 predicted targets showed high enrichment in cell death and survival (70 molecules; *p-value range 4.83E-04 - 3.62E-09*), cell cycle (43 molecules; *p-value range 4.87E-04 - 5.14E-09*), cellular assembly and organization (37 molecules; *p-value range 4.08E-04 - 1.28E-08*), and cellular development (62 molecules; *p-value range 4.95E-04 - 3.81E-09*), which supports previous findings and the role of these miRNA clusters in self renewal, cell proliferation and cell cycle progression of human ESC and iPSCs [18, 19]. The upstream regulator prediction of the miR-302 and miR-371-373 clusters and miR-92b mRNA targets showed inhibition (activation *z-score* ≤ *-2.0*) of several cytokines (*IL4, CSF2, CD40LG, IL2, IL18, IL7, IFNB1, IFNG* and *OSM*), transcription regulators (*STAT1, RUNX3, XBP1, IRF1, EGR1* and *PDX1*) and the growth factor *BMP4*, which suggests the role of these miRNA clusters in inhibiting the LCL specific pathways and pathways involved in stem cell differentiation.

Role of DE miRNAs in the regulation of key LCL and iPSC canonical pathways

Further, we explored the miRNA-mRNA interactions in key LCL and iPSC canonical pathways.

B cell receptors signaling: Previous studies have shown that latently expressed EBV oncoproteins mimic B-cell receptor (BCR) signaling to activate NF-κB, JNK, and MAPK pathways and support rapid growth and survival of latently infected B-cells/LCLs [3, 5] and these pathways are significantly inhibited upon reprogramming of LCLs into iPSCs [2]. The miRNA target prediction analysis identified 36 DE mRNAs associated with BCR signaling pathways, which were the predicted targets for 34 DE miRNA families, resulting in a total of 61 miRNA-mRNA interactions potentially involved in the regulation of BCR signaling. The most significant 53 miRNA-mRNA interactions, out of which 24 showed opposite expression of miRNA and their target mRNA, are shown in **Figure 4**.

Human ESC pluripotency: We have shown previously that LCL reprogrammed iPSCs have similar properties both in self renewal and differentiation capacity to human ESCs [2]; there-

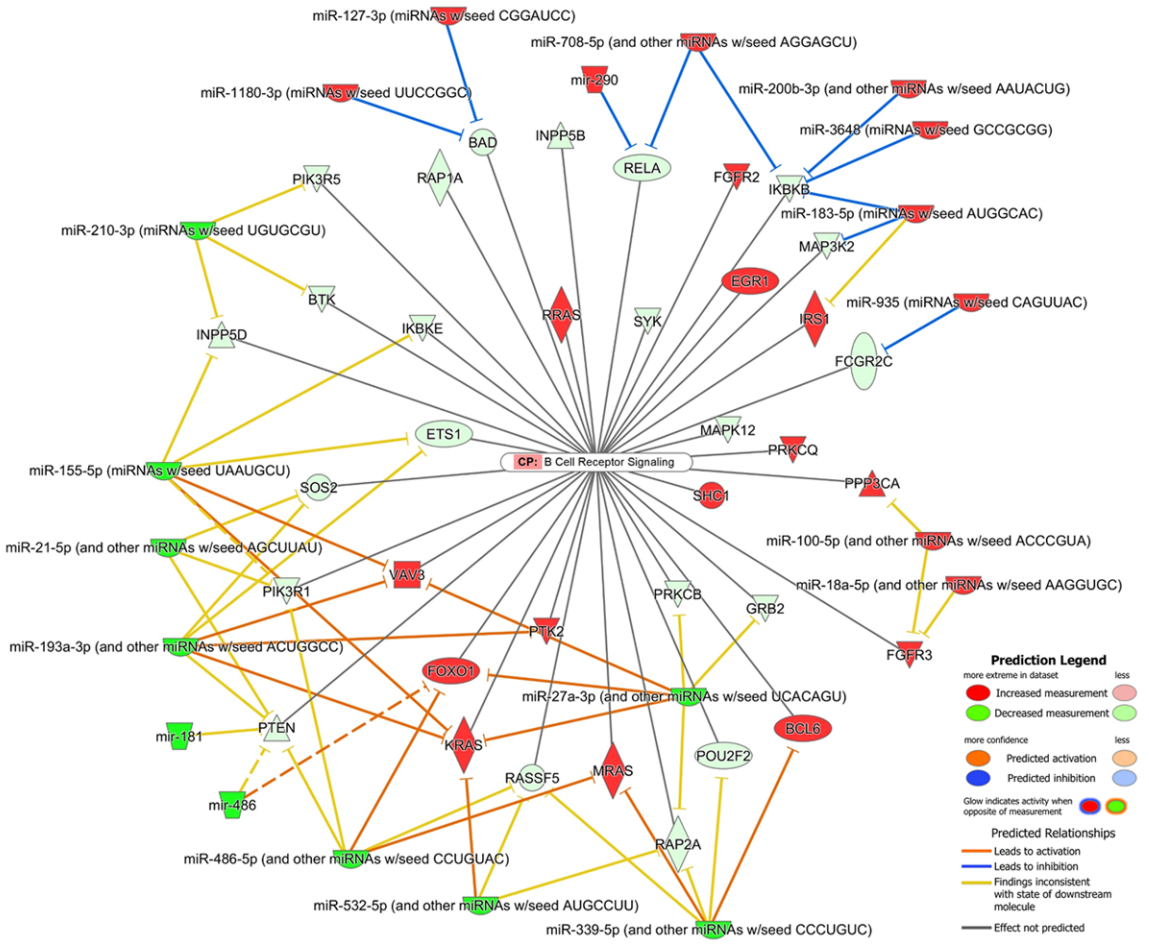

Figure 4. miRNA-mRNA network showing potential miRNA-mRNA interactions in B-cell receptor signaling.

fore next we explored miRNA-mRNA interactions between DE mRNA associated with human ESC pluripotency pathways. A total of 46 interactions between 24 miRNA families and 27 DE mRNAs were identified (**Figure 5**). About 50% of these miRNA-mRNA interactions showed opposite expression pairing of the miRNAs and their targets.

Conclusions

We identified 85 miRNAs that were DE between LCLs and their reprogrammed iPSCs. These 85 DE miRNAs mapped to 67 mature miRNA families. The 1,842 mRNAs that were DE between LCLs and their reprogrammed iPSCs were predicted (with high confidence) to be the potential target of the 52 mature miRNA families identified in this study. A total of 2,642 miRNA-mRNA interactions were identified between LCLs and iPSCs DE miRNome and transcriptome which suggests a significant role of miRNA mediated regulation of transcriptional changes that takes place during reprogramming of LCLs into iPSCs. There are several potential limitations of this study. For example, only a small percentage of these miRNA-mRNA interactions are experimentally observed, and the majority of the predicted interactions are yet to be confirmed, only about half of the interactions were between oppositely expressed miRNA-mRNA pairs, and diverse genetic and epigenetic mechanisms are involved in transcriptional regulation during iPSC reprogramming of LCLs, which complicates the identification of potential miRNAs targets. However, this study provides a comprehensive list of miRNA-mRNA interactions predicted with high confidence using genome wide DE miRNome and transcriptome of LCLs and their reprogrammed iPSCs. This data is relevant to better understand EBV biology in LCLs and in EBV related lymphomas, as well as

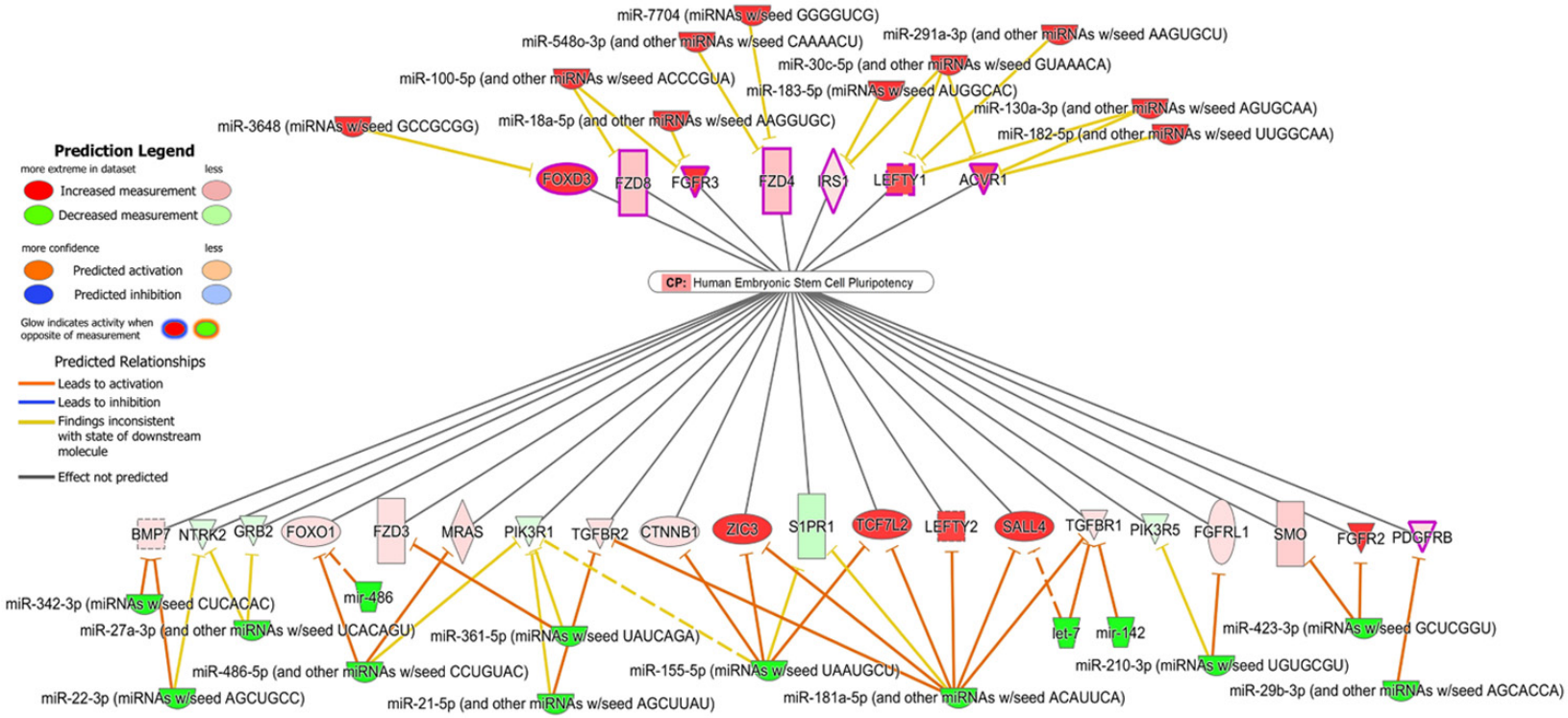

Figure 5. miRNA-mRNA network showing potential miRNA-mRNA interactions in human embryonic stem cell pluripotency pathways.

understanding the miRNA mediated transcriptomic regulation of iPSC self-renewal and pluripotency.

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Disclosure of conflict of interest

None.

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