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Unraveling The Effects of *DICER1* Overexpression on Immune-Related Genes Expression in Mesenchymal Stromal/Stem Cells: Insights for Therapeutic Applications

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Abstract

Objective: The immunoregulatory properties of mesenchymal stromal/stem cells (MSCs) bring a promise for the treatment of inflammatory diseases. However, their ability to suppress the immune system is unstable. To enhance their effectiveness against immune responses, it may be necessary to manipulate MSCs. Although some dsRNA transcripts come from invading viruses, the majority of dsRNA has an endogenous origin and is known as endo-siRNA. DICER1 is a ribonuclease protein that can generate small RNAs to modulate gene expression at the post-transcriptional level. We aimed to evaluate the expression of several immune-related genes at mRNA and protein levels in MSCs overexpressing DICER1 exogenously.

Materials and Methods: In this comparative transcriptomic experimental study, the adipose-derived MSCs (Ad-MSCs) were transfected using the pCAGGS-Flag-hsDicer vector for the *DICER1* overexpression. Following the RNA extraction, mRNA expression level of *DICER1* and several inflammatory cytokines were examined. We performed a relative real-time polymerase chain reaction (PCR) assay and transcriptome analysis between two groups including DICER1-transfected MSCs and control MSCs. Moreover, media from the transfected MSCs were evaluated for various interferon response factors by ELISA.

Results: The overexpression of *DICER1* is associated with a significant increase in the mRNA expression level of *COX-2, DDX-58, IFIH1, MYD88, RNase L, TLR3/4*, and *TDO2* genes and a downregulation of the TSG-6 gene in MSCs. Moreover, the expression levels of *IL-1, 6, 8, 17, 18, CCL2, INF-γ, TGF-β*, and *TNF-α* were higher in the DICER1-transfected MSCs group.

Conclusion: It seems that the ectopic expression of *DICER1* in Ad-MSCs is linked to alterations in the expression level of immune-related genes. It is suggested that the manipulation of immune-related pathways in MSCs via the *Dicer1* overexpression could facilitate the development of MSCs with distinct immunoregulatory phenotypes.

Keywords: DICER1, Immunomodulation, Mesenchymal Stromal/Stem Cells, RNA-Sequencing

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Introduction

Mesenchymal stromal/stem cells (MSCs) are a category of stem cells deemed to have a high potential for therapeutic stem cell applications and regenerative medicine (1). MSCs are a diverse population of cells that possess the ability to self-renew and differentiate into various mesenchymal lineages, such as cartilage, adipose tissue, and bone (2). There is enough evidence that MSCs have immunomodulatory properties and can modify immune responses and inflammatory environments (1).

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The immunosuppressive function of MSCs is not stable, and they need to be activated for their immunosuppressive properties to be unlocked. MSCs are now referred to as "inflammation sensors" due to their interaction with the inflammatory environment and immunomodulatory properties. They can affect the immune system through direct cell-to-cell attachment, inflammatory factor secretion, and programmed cell death induction (2). There is a growing interest in finding new ways to enhance the therapeutic potential of MSCs in various clinical conditions.

Given that miRNAs are key regulators of gene expression and that MSC functions are heavily dependent on their transcriptome profiles. Therefore, it is reasonable to hypothesize that miRNAs could have a significant impact on the immunomodulatory properties of MSCs. Understanding these interactions may provide a manipulated miRNA profile that brings a new chance of MSC-based therapy. The miRNAs are produced from pri-miRNAs, which are generated by the RNA polymerase II in the nucleus, and then processed to pre-miRNAs and exported to the cytoplasm (3).

The Dicer protein is a multi-domain endoribonuclease that mainly known for its catalytic function in regulating gene expression and specificity for processing endogenous dsRNAs (4). This highly conserved RNase has a crucial role in various physiological and molecular pathways during development and stress resistance (5). Abnormal expressions of Dicer were demonstrated in different types of malignancies, with an increased expression found in the prostate, colorectal, and early lesions of lung adenocarcinoma and decreased expression found in breast and invasive lung adenocarcinoma (6-9). The dysregulation of Dicer is associated with poor survival, invasion, and metastasis (8, 10, 11). Most of the available data on the abnormal expression of Dicer is related to cancer cell lines, and there is not enough data about the role of Dicer in human stem cells. The under or overexpression of Dicer results in the differentiation potential of stem cells for miRNA biogenesis (12). The probable role of Dicer in the regulation of immunological properties of human stem cells has not been previously discussed, but it has been demonstrated that Dicer-deficient murine stem cells showed inappropriate proliferation and fail to express pluripotency markers (13).

Regarding the therapeutic potential of MSCs, it has been suggested that manipulation of these cells toward an enhancement of their therapeutic functions, may increase their efficacy in treating human diseases. Therefore, this study aimed to over-express the *DICER1* in human adipose-derived MSCs (Ad-MSCs) and investigate the effect of the ectopic expression of this gene on the expression of immune-related genes.

Material and Methods

The biomedical research Ethics Committee ACECR, Khorasan Razavi, Iran, approved this investigation (IR.ACECR.JDM.REC.1398.007). This study was performed on samples obtained from 3 healthy females who underwent a plastic surgery in the Day Clinic, (Khorasan Razavi, Iran). All participants signed an informed consent.

Isolation and culture of human MSCs derived from adipose tissue

Adipose-derived stromal/stem cells were provided by fresh, healthy lipoaspirate of patients who underwent a plastic surgery. In our lab, samples were washed with the phosphate-buffered saline buffer (PBS, Biowest, Germany), containing 0.1% penicillin-streptomycin (pen-strep, 15140122, Thermo Fisher Scientific, USA). They were then incubated in the presence of 0.1% collagenase type I (17100, Gibco, USA) at 37°C for one hour, followed by inactivation of the collagenase with the 10% fetal bovine serum (FBS, 11960044, Gibco, USA). After a centrifugation (600 g for 10 minutes) to remove adipose cell debris, the pellets were suspended in the Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA), containing 10% FBS and 0.1% pen-strep. The cells were incubated in a 5% CO_2 at 37°C and the culture medium was changed every three days. Experiments were conducted using cells at passage number 3 from the lipoaspiration.

Flow cytometry analysis of Ad-MSCs surface markers

To confirm the purity and characterization of isolated Ad-MSCs, we investigated the (RNA or protein) expression of some specific mesenchymal stem cell surface markers, such as CD44, 73, 90, and 105, and also the hematopoietic cell-specific markers, including CD11b, CD34, and CD45 as. Briefly, 10^6 Ad-MSCs were suspended in the 250 µl cold PBS with 5% FBS and incubated at room temperature with the 1 µl of interested antibodies (BioLegend, Spain) for 1 hour in the dark. Then the expression of surface antigens was evaluated by a FACSCalibur flow cytometer (BD Bioscience, USA) and data were analyzed by FlowJo 7.6 software (Tree Star, Ashland, OR) (14).

Plasmid construction and cell transfection

The pCAGGS-Flag-hsDicer vector, including the human *DICER1* gene sequence, was obtained from Addgene (41584, USA). Then, the fluorescent ZsGreen marker-2A-Puromycin fragment inframe was derived and amplified from the pCDH-513b lentivirus vector (513B-1, System Biosciences, USA) and cloned in the pCAGGS-Flag-hsDicer vector (Fig.1). This cistronic construct simultaneously expresses *DICER1* was connected to the fluorescent ZsGreen marker and puromycin by the 2A peptide. Moreover, the pCDH-513b vector was applied as a control for transfection experiments (approximately up to 90% transfection efficiency in cells). All vectors were purified by using the QIAGEN Plasmid Maxi Kit (12162., USA), according to the manufacturer's instructions. MSCs were transfected by using the Lipofectamine 3000 reagent (L3000001, Thermo Fisher Scientific, USA)

according to the manufacturer's protocol. Briefly, the cells in several groups were transfected with 2500 ng (per 6-well plate) of the pCAGGS-Flag-hsDicer plasmid and a plasmid expressing the fluorescent ZsGreen marker as a control group, separately. The MSCs were selected with 2 ug/ml puromycin (A1113803, Thermo Fisher Scientific, USA) 48 hours post-transfection and the remaining cells were selected after 72 hours of treatment with puromycin.

RNA extraction and quantitative real-time polymerase chain reaction analysis

The extraction of total RNA, removal of DNA contamination using DNase I treatment, and subsequent cDNA synthesis were carried out using the Tripure reagent from Roche (Roche, Germany), the DNase Removal kit, and MMuLV Reverse Transcriptase from Thermo Fisher Scientific (Thermo Fisher Scientific, USA), respectively. All procedures were performed according to the manufacturers' instructions. Real-time PCR was performed by SYBR Green PCR Master Mix (A323402., AMPLIQON, Denmark) on a CFX-96 machine (Bio-Rad, USA) with specific primers for the candidate genes and data were normalized by the *RPLP0* housekeeping gene. The specific primer sequences and amplicon size of genes are demonstrated in the supplementary file. The fold change between 0.5 and 2 was interpreted as a normal expression. Since the efficiency of all primers was measured to be 90 to 110%, the $2^{-\Delta\Delta CT}$ method was utilized to compare the relative expression. The gene expression at the mRNA level was evaluated. Our selected genes were included , *COX-2* (a proliferation-related transcriptional factor), *DDX-58* (an antiviral innate immune response receptor RIG-I), *IFIH1* (an interferon-induced helicase C domain-containing protein), *MYD88* (a myeloid differentiation primary response), *RNase L* (an interferon-induced ribonuclease which destroys all RNA within both cellular and viral), *TLR3* (an RA-related important pattern recognition receptor), *TLR4* (a bacterial lipopolysaccharide pattern recognition receptor), *TDO2* (a systemic regulator of tryptophan levels).

Enzyme-linked immunosorbent assay

Concentrations of the secreted cytokines including IL-1, 4, 6, 8, 10, 17, 18, 23, INF-γ, TNF-α, TGF-β, and CCL2, in the supernatant of the Dicer-transfected MSCs were measured with the use of ELISA kit (KPG, Iran) according to the manufacturer's instructions. Briefly, the supernatant of transfected and non-transfected cells was added to the ELISA strip plates that were pre-coated with captured antibodies specific of each cytokine, and colorimetric absorbance was recorded at a wavelength of 450 nm. The MSCs were transfected with the GFP expressing vector, pCDH-513b plasmids, and non-transfected cells were applied as control groups.

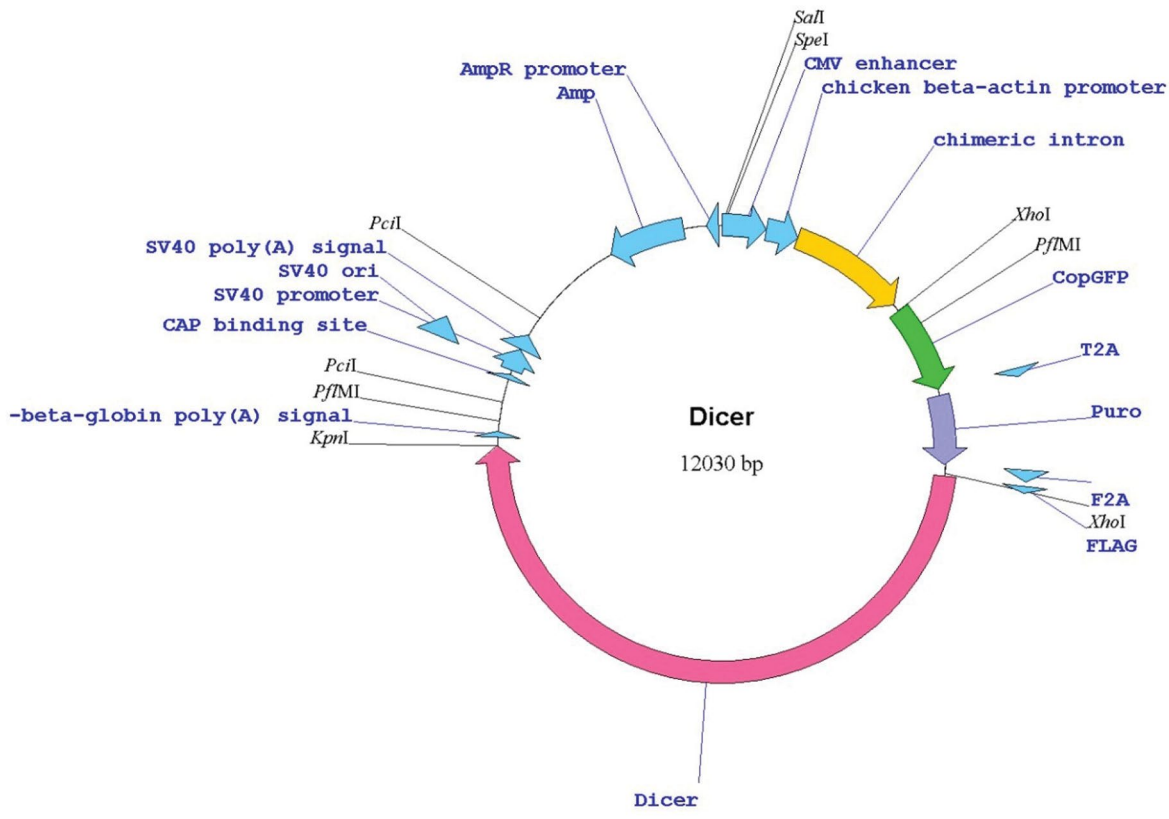


Fig.1: The genetic map of the pCAGGS-Flag-hsDicer construct that simultaneously express *DICER1* connected by a 2A peptide to the fluorescent ZsGreen marker.

RNA-sequencing by Illumina NovaSeq6000

RNA sequencing, mapping, and normalization were performed as previously described (15). Briefly, sequencing libraries were produced from 100 ng RNA sample, using KAPA RNA HyperPrep Kit (Kapa Biosystems, USA) with RiboErase (HMR) kit (Hoffmann-La Roche, Switzerland).

Then, paired-end reads (2×100 nucleotides) were attained for 4 samples, including 2 RNA samples of *DICER1* overexpressed MSCs and 2 of fluorescent ZsGreen markers overexpressed MSCs, on the Illumina NovaSeq 6000 platform (CeGaT company, Germany). The sequencing reads were aligned to the GRCh38 (hg38) human reference genome, using STAR 2.7.1, and the HTSeq package was run under the union mode. The differential expression of genes was identified by the DESeq2 package in R software from Bioconductor. The total RNA-sequencing data were deposited in the NCBI database under accession number (PRJNA681026).

Bioinformatics analysis

The GeneMANIA database (http://www.genemania.org) was used to identify the gene-to-gene interactions network and target genes correlated to DICER protein and immune genes. We also used NetworkAnalyst webserver (https://www.networkanalyst.ca) to find the expression network of differentially expressed genes (DEGs) and the most closely associated genes within the co-expression network and physical interaction.

Statistical analysis

The IBM SPSS 22 statistical software (La Jolla, USA) and GraphPad Prism 5.0 (La Jolla, USA) were used to perform the data analysis and figure drawing, respectively. A $P<0.05$ was considered to indicate a statistically significant difference. A One-Sample t test was used to compare the log2 fold change of qRT-PCR results.

Results

Characterization of Ad-MSCs analysis with flow cytometry

The MSCs derived from the adipose tissue were verified with their spindle-like shape examination and specific surface markers were identified. The expression levels of CD90, 44, 105, and 73 were 98.5, 98.2, 90.8, and 97.7%, respectively, whilst the expression of non-mesenchymal stem cell-specific markers, including CD11b, 45, and 34 were 4.22, 0.88, and 2.12%, respectively (Fig.2).

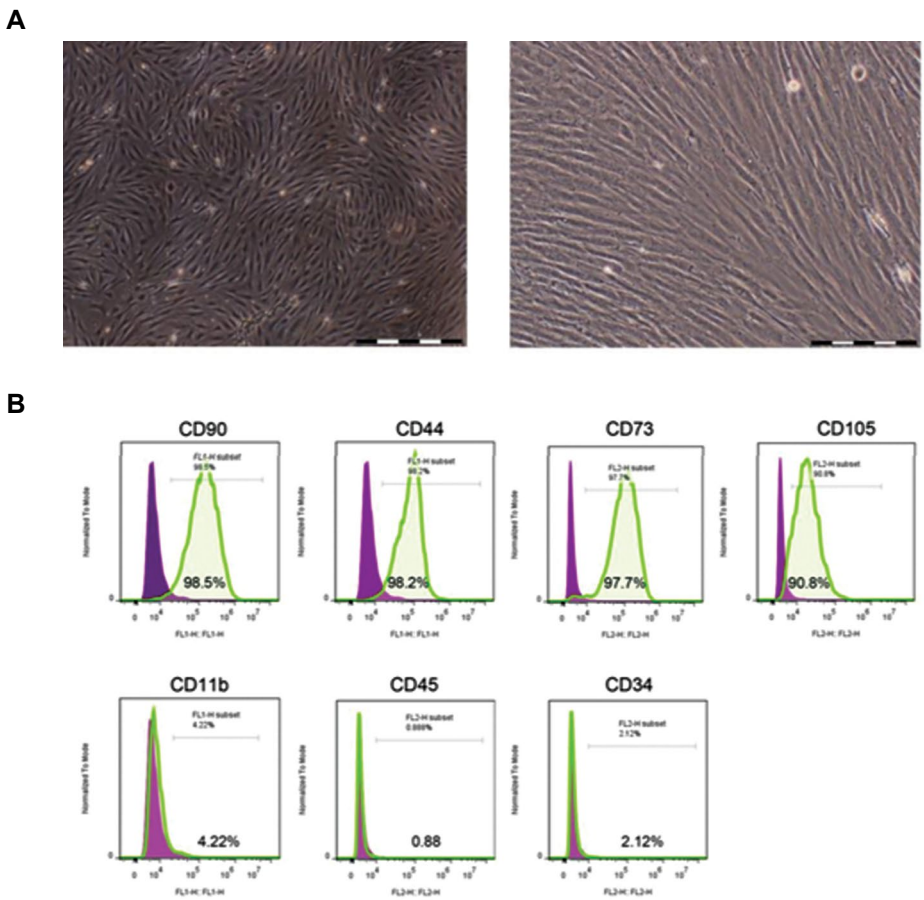


Fig.2: Characterization of human Ad-MSCs. **A.** Bright field images of cells at different magnifications showing a fibroblast-like morphology (scale bar: 500 µm (left) and 200 µm (right). **B.** Flow cytometry analysis shows almost all cultured Ad-MSCs (more than 90%) markers, including CD90, CD44, CD73, and CD105 expression, whereas a small portion of the cells expressed CD11b, CD45, and CD34 markers. We illustrated expressions of cell surface markers of Ad-MSCs that were compared with their respective isotype controls.

Overexpression of *DICER1* in MSCs

Using fluorescent microscopy, the transfection efficiency of the pCAGGS-Flag-hsDicer construct was compared with the pCDH513b fluorescent ZsGreen marker, following *DICER1* overexpression in MSCs. The transfection results of the pCAGGS-Flag-hsDicer and pCDH513b control transfected MSCs are revealed in Figure 3. Significant overexpression of *DICER1* was detected in the pCAGGS-Flag-hsDicer transfected MSCs in comparison with the pCDH513b control MSCs.

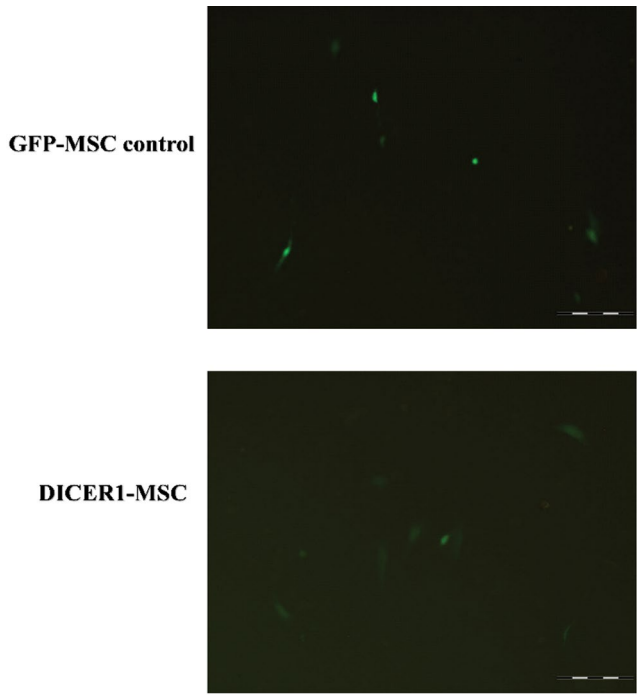


Fig.3: Ectopic expression of *DICER1* in MSCs. Fluorescence microscopy images of Ad-MSCs overexpressing pCDH-513b vector as a control (top) and also cells with ectopic expression of pCAGGS-Flag-hsDicer (bottom) (scale bar: 200 µm).

Overexpression of *DICER1* involved in the MSC-mediated immune regulation

By confirming the *DICER1* overexpression in Ad-MSCs, we evaluated the mRNA expression levels of 10 pro and anti-inflammatory cytokine genes by the qRT-PCR technique after triplicate examinations (Fig.4). Overexpression of *DICER1* led to a significant increase (P<0.0001) in the levels of mRNA expression of some genes, including *COX-2*, *DDX-58*, *IFIH1*, *MYD88*, *RNase L*, *TLR3*, *TLR4 and TDO2* (up to 3, 6, 4, 4.5, 3, 16, 4, 3 folds, respectively) in comparison with to the control cells. In addition, the overexpression of *DICER1* resulted in a significant reduction (P<0.05) in the expression of mRNA levels of *TSG-6*. The mRNA levels of *TSG-6* and *INF-γ* were decreased to 2.2 and 2 folds, respectively.

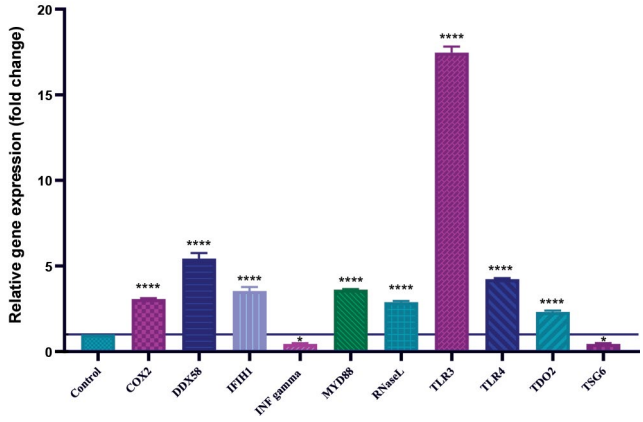


Fig.4: Ectopic expression of *DICER1* significantly impacts the mRNA expression of several immune-related genes in MSCs. The expression of all genes in the control group (GFP-MSCs) was set to a value of 1. When compared, the expression levels of these genes in Ad-MSCs with overexpressed *DICER1* gene were reported as relative values compared to the control group. RPLP0 was used as an intron control. The line parallel to the x-axis passes through one and the overexpression and downregulation of these genes in ASCs transfected with DICER1 were then compared with the control group. *; P<0.05 and ****; P<0.0001

To investigate the amount of pro and anti-inflammatory cytokines at the protein level of MSCs overexpressing *DICER1*, we determined the concentration of the secreted cytokines in each group's culture media by ELISA assay. The concentration of IL-4 (12.58 pg/mL), IL-10 (34.48 pg/mL), and IL-23 (24.32 pg/mL) were non − significantly less in the *DICER1*-transfected MSCs than the control group, whilst the concentrations of IL-1, 6, 8, 17, 18, CCL2, INF-γ, TGF-β, and TNF-α were higher in the *DICER1*-transfected MSCs group (28.19, 113.6, 156.2, 19.5, 42.44, 30.43, 25.12, 21.17, 45.45 pg/mL), respectively (Fig.5).

RNA-sequencing of *DICER1* overexpressing MSCs

The transcriptomes of o ur genetically modified MSCs were analyzed to determine if there were any changes in its gene expression and dsRNA related pathways. RNA sequencing was performed on the total RNA that was extracted from the duplication of MSC-GFP and MSC-DICER1 cells to identify the gene expression pattern. Our analysis revealed 527 significant DEGs between MSC-GFP and MSC-DICER1 cells (adjusted P<0.05, -1 <log2 Fold change <1). The most enriched gene terms in molecular function (MF) were extracellular matrix structure, organization, protein folding chaperone, cofactor binding/signaling receptor activator/receptor-ligand activity, and cell adhesion molecule binding, as shown in Figure S1A (See Supplementary Online Information at www.celljournal.org).

The metabolic processes of RNA molecules, including rRNA, mRNA, and ncRNA, as well as ribosome biogenesis, were found to be the most enriched in the biological process (BP) category (Fig.S1B, See Supplementary Online Information at www.celljournal.org). We did not

find an inclusive list of DEGs. Therefore, we evaluated the expression of some selected genes via qRT-PCR experiments. We concluded that the overexpression of some of genes, including *CDSN*, *POSTN*, and *HELLPAR*, plays a role in the stemness maintenance. Furthermore, *LCE1F*, *NTSR1*, *EPHA7*, *LRR1*, *ANKRD28* and *ZBTB12* were some of the most suppressed genes in our study. Most of the downregulated genes were involved in signal transduction pathways.

The significance of molecular function and BP is demonstrated in Table S1 (See Supplementary Online Information at www.celljournal.org).

Our analysis revealed that these DEGs are primarily involved in various biological processes, including the innate immune system, interferon response types α/β/γ, Toll-like receptor signaling, transport of mature mRNA independent of SLBP, transport of sugars, bile salts, organic acids, metal ions, and amine compounds, cytosolic sensing of pathogen-associated DNA, Th17 cell differentiation, NAD metabolism, PEDF signaling, ERK signaling, responses to bacterial infections, G-protein signaling via Ras family GTPases in kinase cascades, replication of viral genome, and ATM-dependent DNA damage response.

Although, the whole list of immunity related genes has not been found in the RNA-seq data, we considered a panel of genes to evaluate by qRT-PCR and ELISA at mRNA and protein levels. This panel contained the interferon response pathway genes, particularly those genes that were involved in the interferon response, α and/or β and/or γ types, ERK signaling, and Toll-like receptors. The results have demonstrated the dysregulation of innate immune response genes of the interferon response pathway. These genes included *IL-1*, *4*, *6*, *8*, *10*, *17*, *18*, *23*, *INF-γ*, *TNF-α*, *TGF-β*, *CCL2*, *COX-2*, *DDX-58*, *IFIH1*, *MYD88*, *RNase L*, *TLR3*, *TLR4*, *TDO2*, and *TSG-6*.

Our gene ontology analysis of mesenchymal stromal stem cells overexpressing *DICER1* showed that *DICER1* overexpression can affect various cellular processes. Particularly, we observed changes in heterochromatin-modifying enzymes, catalytic activity on RNA, and the formation of ribonucleoprotein complexes. These findings suggest that an immune function dysregulation may be an indirect result of the molecular changes associated with the DICER1 protein overexpression (Fig.6).

Gene co-expression network analysis

All *DICER1*, pro and anti-inflammatory cytokines and immunoregulatory genes were imported into the GeneMANIA database to generate the protein-protein interaction (PPI) network. The genes interaction was based on pathways, physical interactions, and genes co-expression. The data defined the biological functions of genes through the co-expression network. Our analysis revealed that the *DICER1* gene interacts with several gene markers, including *TARBP2*, *PRKRA*, and *ERCC3*, but not with cytokines. Moreover, anti-inflammatory and pro-inflammatory mediators interacted with several genes, including *TCN1*, *GNA1/2*, *PF4*, *GATA3*, *CBLAF*, *CUBN*, *IQGAP1*, *MAVS*, *CYLD*, *IRF5/7*, *TIRAP*, *LY96*, *IRAK1/2*, *TICAM2*, *IDO1/2*, *ECSIT*, and *MBL2*. These results confirmed the biological relevance between *DICER1* and genes expression of inflammatory cytokines (Fig.S2, See Supplementary Online Information at www.celljournal.org). The Network analysis revealed that NCBP1a protein was among the upregulated nodes. While, the NCBP1a protein is an mRNA-cap binding protein known for its role in transcription regulation by RNA polymerase II. Additionally, many genes associated with immune response were downregulated, in agreement with our earlier discussion. Our network analysis revealed that the NCBP1, an mRNA-cap binding protein, that is involved in transcription regulation by RNA polymerase II, was among the upregulated nodes. Additionally, many genes related to immune response were found to be downregulated, as previously discussed.

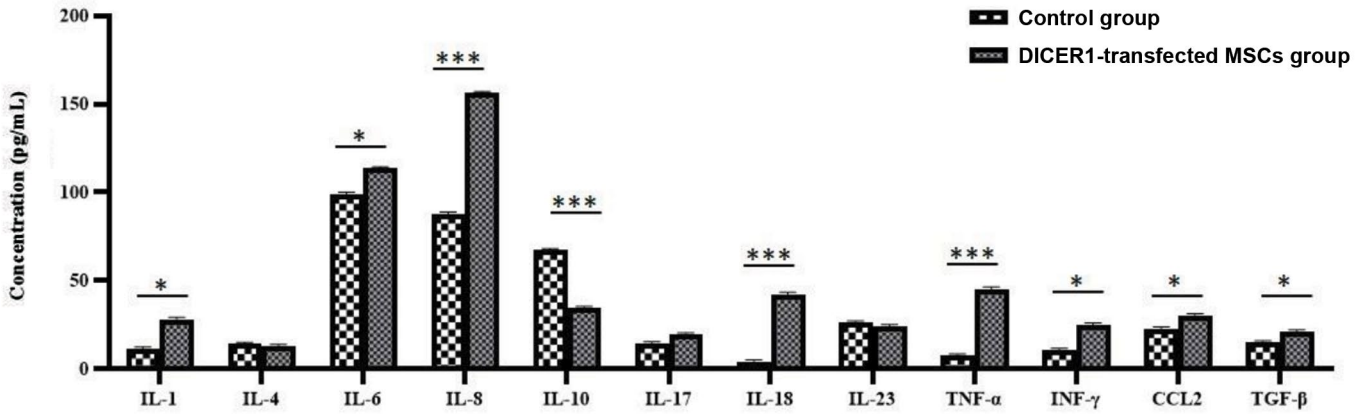
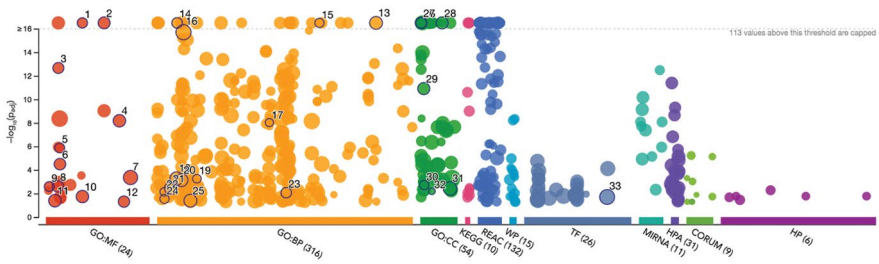


Fig.5: Cytokine expression analysis at protein level. The results indicate a significant difference in the expression of several cytokines, including IL-1, IL-6, IL-8, IL-10, IL-18, TNF-α, IFN-γ, CCL2 and TGF-β between DICER1-MSCs and the control group. Of these cytokines, only IL-10 was overexpressed in the control group in comparison with the DICER1 overexpressing cells. *; P<0.05 and ***; P<0.001.

A



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3	GO:MF	GO:0005201	extracellular matrix structural constituent	2.139×10^{-13}
4	GO:MF	GO:0050839	cell adhesion molecule binding	6.536×10^{-9}
5	GO:MF	GO:0005518	collagen binding	1.329×10^{-8}
6	GO:MF	GO:0008017	microtubule binding	3.178×10^{-5}
7	GO:MF	GO:0097367	carbohydrate derivative binding	4.321×10^{-4}
8	GO:MF	GO:0005125	cytokine activity	2.513×10^{-3}
9	GO:MF	GO:0003777	microtubule motor activity	2.536×10^{-3}
10	GO:MF	GO:0030545	signaling receptor regulator activity	1.808×10^{-2}
11	GO:MF	GO:0004672	protein kinase activity	4.071×10^{-2}
12	GO:MF	GO:0061134	peptidase regulator activity	4.837×10^{-2}
13	GO:BP	GO:1903047	mitotic cell cycle process	8.712×10^{-28}
14	GO:BP	GO:0006334	nucleosome assembly	1.646×10^{-27}
15	GO:BP	GO:0071459	protein localization to chromosome, centromer...	1.420×10^{-19}
16	GO:BP	GO:0007275	multicellular organism development	1.969×10^{-16}
17	GO:BP	GO:0045653	negative regulation of megakaryocyte different...	9.346×10^{-9}
18	GO:BP	GO:0006259	DNA metabolic process	5.209×10^{-4}
19	GO:BP	GO:0010575	positive regulation of vascular endothelial grow...	5.468×10^{-4}
20	GO:BP	GO:0006954	inflammatory response	7.201×10^{-4}
21	GO:BP	GO:0003013	circulatory system process	3.619×10^{-3}
22	GO:BP	GO:0002227	innate immune response in mucosa	7.166×10^{-3}
23	GO:BP	GO:0050830	defense response to Gram-positive bacterium	8.448×10^{-3}
24	GO:BP	GO:0002251	organ or tissue specific immune response	2.830×10^{-2}
25	GO:BP	GO:0009617	response to bacterium	4.033×10^{-2}
26	GO:CC	GO:0000786	nucleosome	5.419×10^{-40}
27	GO:CC	GO:0000775	chromosome, centromeric region	4.123×10^{-26}
28	GO:CC	GO:0062023	collagen-containing extracellular matrix	3.222×10^{-21}
29	GO:CC	GO:0005819	spindle	1.209×10^{-11}
30	GO:CC	GO:0005871	kinesin complex	1.895×10^{-3}
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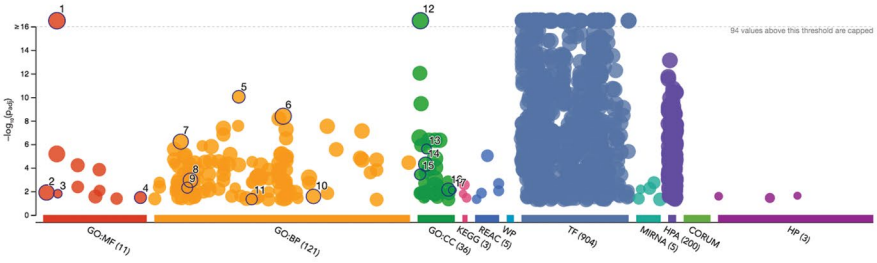

ID	Source	Term ID	Term Name	p_{adj} (query_1)
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2	GO:MF	GO:0003824	catalytic activity	1.247×10^{-2}
3	GO:MF	GO:0008171	O-methyltransferase activity	1.578×10^{-2}
4	GO:MF	GO:0140098	catalytic activity, acting on RNA	3.337×10^{-2}
5	GO:BP	GO:0034660	ncRNA metabolic process	9.153×10^{-11}
6	GO:BP	GO:0050794	regulation of cellular process	4.094×10^{-9}
7	GO:BP	GO:0007275	multicellular organism development	5.936×10^{-7}
8	GO:BP	GO:0010033	response to organic substance	1.144×10^{-3}
9	GO:BP	GO:0009451	RNA modification	4.860×10^{-3}
10	GO:BP	GO:0070887	cellular response to chemical stimulus	2.692×10^{-2}
11	GO:BP	GO:0042180	cellular ketone metabolic process	4.623×10^{-2}
12	GO:CC	GO:0005737	cytoplasm	2.451×10^{-17}
13	GO:CC	GO:0030684	preribosome	2.512×10^{-8}
14	GO:CC	GO:0030054	cell junction	4.883×10^{-5}
15	GO:CC	GO:0005681	spliceosomal complex	3.649×10^{-4}
16	GO:CC	GO:0099081	supramolecular polymer	7.067×10^{-3}
17	GO:CC	GO:1990124	messenger ribonucleoprotein complex	7.829×10^{-3}

version	e109_eg56_p17_1d3191d
date	4/22/2023, 11:50:17 AM
organism	hsapiens

Fig.6: The gene set enrichment analysis was performed using the g: Profiler web server on a set of differentially expressed genes identified in the *DICER1*-overexpressing MSCs. The top enriched gene ontology (GO) terms are displayed in a bubble plot, with the color indicating their level of significance, as measured by the -log10 of the P value. The size of each bubble represents the number of genes in the term. The x-axis shows different GO categories based on molecular function (MF), biological process (BP), and cellular components (CC), as well as different databanks. The y-axis shows the significance of the term, measured by the adjusted P value (q value). **A.** The pathway analysis of overexpressed genes revealed significant enrichment in several biological processes, including 'chromatin remodeling', 'cytokine activity', and 'extracellular matrix'. **B.** Downregulated genes involving in pathways such as 'RNA modification', 'spliceosome complex', and 'ncRNA metabolic process' are among the suppressed pathways.

Discussion

MSCs have been a promising target for cell regenerative therapy of numerous immune-mediated diseases and immune homeostasis due to their desirable characteristics, such as immunoregulatory features, multi-lineage differentiation ability, and directed tissue homing (16). Today, stem cell therapy is a fast-growing field, while the MSC draws attention to its potential. It seems that MSCs are safer and more easily applicable than stem cells from different sources. While the use of stem cells has shown effectiveness in treating various clinical conditions, transplantation of stem cells carries the risk of heterogeneity, genomic and chromosomal instability, as well as potential tumorigenesis *in vivo*. Although unfavorable outcomes are primarily caused by environmental host factors, enhancing the functions of MSCs through gene manipulation may help to overcome many of the shortcomings associated with transplantation (17).

The microenvironment surrounding MSCs influences the signalling pathway and cell polarization, leading to the secretion of potent soluble cytokine factors that prompt cells to adopt immunomodulatory and anti-inflammatory phenotypes. Additionally, this condition activates the immunosuppressive functions of MSCs, which has a critical role in their biological functions such as tissue repair (18). Several studies have evaluated the role of *DICER1* expression in stem cells. It has been demonstrated that the *DICER1* deficiency is linked to the type I interferons and PKR activation in murine embryonic stem cells (mESCs), making these cells more susceptible to the antiviral responses induced cytotoxicity that may be probably through a miRNA biogenesis deficiency. While the NF-κB pathway is the main factor in controlling the expression of interferons, the altered expression of some miRNAs, such as the miR-290 cluster, could be the reason for activating the NF-κB pathway in mESCs with the *DICER1* deficiency (13). Our findings support previous research showing that overexpressing DICER1 results in increased activity of the NF-κB pathway. Similar to its role in neural crest-derived stem cells, NF-κB acts as a key regulator of differentiation in MSCs, inhibiting the development of muscle and cartilage cells (19). It is noteworthy that this differentiation could be attributed to the inhibition of NF-κB signaling pathway, which leads to the counteraction of pro-inflammatory conditions (20). However, such a role is more likely to be context-dependent, as some studies reported that chondrogenesis could be induced without dysregulation even after blocking the NF-κB pathway (21).

Activation of NF-κB can lead to the proliferation, adhesion, and migration of MSCs. It can also trigger inflammatory responses and the production of cytokine such as IL-1, 2, 6, 8, 12, and TNF-α from these cells (22, 23).

It is noteworthy that the immunosuppressive properties of MSCs are related to their secretion factors, such as ILs, TGF-β, INF-γ, and TNF-α (24). Moreover, the silencing of *DICER* is linked to apoptosis and cell proliferation inhibition in myeloma and leukemia (6). The dysregulation of DICER has been associated with cell proliferation and tumor development in lung cancers (10). The decreased expression of *DICER* during aging and the development of age-related insulin resistance highlights its metabolic effect role in the adipose tissue (25).

We hypothesized that the overexpression of *DICER1* in MSCs may cause immune responses. It was suggested that inflammatory cytokines and chemokines, including IL-1, 4, 6, 8, 10, 17, 18, CCL2, TGF-β, INF-γ, and TNF-α, and immunoregulatory genes, including *COX-2*, *DDX-58*, *IFIH1*, *MYD88*, *RNase L*, *TLR3/4*, and *TDO2* may be considered as potential targets for stem cell therapy (24, 26, 27). In our previous study, we assessed the expression of certain immune related genes and found that pre-treating MSCs with B18R protein reduced their inflammatory response when stimulated with poly (I: C) and IFN-γ (28). Hosseini et al. indicated the critical roles of these pro- and anti-inflammatory cytokines and chemokines in the immune suppression (26). It has been confirmed that INF-γ, TNF-α, and IL-1 are crucial factors in initiating cytokine-inducing immunosuppression by MSCs. These cytokines contributing to an inflammatory response can change the immunosuppressive impacts of MSCs (29). Consistent with previous studies, our results indicated that the increase expression of IL-1 could modulate the expression of inflammatory cytokines. A high level of IFN-γ enhances the immunosuppressive capabilities of MSCs through the JAK/STAT1 signaling pathway and IDO1, indicating that cell therapy using MSCs primed with IFN-γ may be effective (30).

Remarkably, MSCs initiate the release of IFN-γ induced by IL-18, which could influence tissue regeneration and offer a new approach for anti-tumor therapy (31). The IL-8 promotes the therapeutic effects of MSCs on the bone regeneration, through the CXCR2-mediated PI3k/Akt signalling pathway (32). The TNF-α considerably suppresses an inflammation occurrence via increasing the levels of TGF-β and IL-10in MSCs (33). The overexpression of IL-10, an anti-inflammatory cytokine, in a normal homeostatic condition, can develop an autoimmune disease through an enhanced inflammatory response. Therefore, detecting key intermediates in various environments that regulate the IL-10 expression is a critical factor for preserving the balance of the immune response (34).

An increased TGF-β expression level acts as a crucial immunosuppressive factor that leads to an immune-modulator function via phosphorylation of Smad2/3, and induces macrophage M2-like polarization to inhibit the excessive inflammatory responses in MSCs (35). The production of IL-6 can result in the creation of anti-inflammatory M2 macrophages, inflammation through the NF-κB pathway, and the activation of transcription through STAT signaling pathways (36). Interestingly,

the CCL-2-secreted MSCs can be involved in therapeutic outcomes by facilitating macrophage repolarization, and result in reducing the inflammatory response through the local employment of macrophages (37). In line with previous studies, our data demonstrated that the IL-17-secreting MSCs promote an immunosuppression function in the cells with the *DICER1* overexpression nature (29). Together, these findings suggest that these cytokines secreted by MSCs may provide a novel approach to therapy by enhancing the therapeutic potential of these cells. In the present study, the overexpression of *DICER1* in Ad-MSCs did not affect the protein expression levels of IL-4 and 23.

Several pathways are involved in the regulation of the immune system through cytokine signalling, including signalling through interferon-α/β/γ, the IL-1/10 family, and the antiviral response triggered by genes stimulated by IFN (38). The increased expression level of *DDX-58*, an innate immune receptor gene, can lead to induce the interferon-α/β expression and the antiviral response was triggered by genes stimulated by IFN, and the IFIH1 (*MDA-5*) gene, which acts as an innate immune receptor, recognized RNA metabolites produced by RNase L, activating the interferon cascade. This is in line with our findings (39).

Given these observations, we propose that MSCs could improve the immunomodulatory properties by increasing the protein expression level of the *TSG-6* gene. Moreover, we hypothesized that the perturbation of cellular immunity by Dicer1 overexpression affects the expression and/or activation of TLRs which leads to trigger innate immune responses via the MYD88-dependent pathway. In this pathway, the activation of NF-κB and MAP kinase may have critical roles in the induction of inflammatory cytokines, such as *IL-1, 6*, and *TNF-α*. Also, the TIR domain-containing adaptor protein-inducing (IFNβ) dependent (TRIF) pathway and induce the type I interferon production (34).

Here, we suggested that the presence of an active stemness behavior and dynamic immunoregulatory nature of MSCs provides a complex context for the prediction of the transcriptional response by modulating the dsRNA response in these cells. The overexpression of *TLR4* can lead to generally overexpression of pro-inflammatory mediators, while *TLR3* induction results in the production of primary immunosuppressive mediators. It is proposed that stimulation of *TLR4* could imitate a pro-inflammatory environment (16). The *COX-2* increased expression level can propose its inhibition by overexpression of the *DICER1* gene that could be a therapeutic strategy for inflammation-mediated disorders (16, 40). Our data may suggest a dysregulation in the inflammatory cytokines and immunoregulatory genes through the expression of *DICER1* in MSCs, which may provide some knowledge of the immunoregulatory potential of these cells.

Conclusion

The overexpression of *DICER1* led to increased expression levels of some inflammatory and anti-inflammatory markers. This dysregulation could be due to inhibition and activation of some inflammatory, anti-inflammatory, and immunoregulatory capacities and alteration of RNA metabolism in human MSCs. It indicates that the *DICER1* manipulation can influence the production of pro-and anti-inflammatory cytokines.

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Authors' Contributions

H.R.B., M.F., H.R.; Designed the experiments. H.R.B., M.F., H.H.; Performed the experiments and collected data. M.F., R.J.E.; Analyzed data. M.M.M.; Performed the RNA sequencing and data analysis. H.R.B., R.J.E., H.H., R.A.M.; Prepared the draft of the manuscript. R.A.M.; Performed the experiments. H.R.; Supervised the study. All the authors approved the final version of the submitted manuscript.

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