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Increased miR-21 expression during human monocyte differentiation into DCs

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1. ABSTRACT

Differentiation of monocytes into dendritic cells (DCs) is characterised by marked changes in gene expression. The role of microRNAs (miRNAs), a new class of small endogenous non-coding regulatory RNAs, in this process is still unclear. We identified miR-223, miR-16, miR-191, miR-24, let-7b, and miR-21 as differentially expressed between monocytes and monocyte derived DCs. We evaluated the expression levels of computationally predicted target genes of miR-21 in human monocytes following stimulation with GM-CSF and IL-4. Moreover, transfection of monocytes with synthetic miR-21 inhibited the expression of a set of genes that were also repressed during monocyte differentiation to DCs in response to GM-CSF and IL-4. Among these, we identified genes that are involved in cell cycle, apoptosis and differentiation such as PDE4B, PDCD4, ANXA1, ING3, STAG2, TGFBI, S100A12, LAT2 and NRIP1. Collectively, the present study highlights the involvement of miRNAs, particularly miR-21 in monocyte differentiation to DCs and identifies potential miR-21 target genes.

2. INTRODUCTION

Monocytes are circulating peripheral blood cells that can be differentiated by cytokines into macrophages, dendritic cells (DCs) as well as into several other cell types such as osteoclast and microglia-like cells (1). DCs constitute a heterogenous population of antigen presenting cells that are critical for bridging the innate and adaptive immunity. Upon pathogen capture, immature DCs receive activation signals, which initiate their maturation and migration to secondary lympoid organs where they activate naïve T cells (2).

Granulocyte macrophage–colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) are commonly used for in the *in vitro* generation of immature myeloid DCs from CD4+ precursors (3). Incubation of these mature DCs with pro-inflammatory mediators such as tumour necrosis factor (TNF), interferon gamma (IFNG), and CD40 ligand (CD154) lead to the generation of fully differentiated cells, known as mature DCs (4). Under physiological conditions, circulating DCs are rare, therefore

the use of *in vitro* culture protocols to generate sufficient DCs from bone marrow cells or blood monocytes for *in vitro* studies and therapeutic applications is well accepted. In this respect, there are ten ongoing clinical trials that use monocyte-derived dendritic cells in vaccine development (5). Therefore, an understanding of the regulation of human monocyte differentiation is highly important from a therapeutic perspective.

Although several studies reported on gene expression in monocytes during DC differentiation (6, 7), the role of microRNAs (miRNAs) in this process is still unclear. MiRNAs are small non-coding RNAs that function as post-transcriptional regulators of gene expression. They act by binding to complementary sites on target mRNAs to induce mRNA cleavage or repression of translation (8). Several studies have shown their involvement in a wide range of developmental processes, including developmental timing, cell proliferation and apoptosis (9). MiRNAs are differentially expressed in immune and non-immune cells and appears to regulate important aspects of innate and adaptive immune responses. Several studies have shown that miRNAs can contribute to immune cell fate and regulate Toll-like receptor and cytokine responses (10, 11). Also, recent data suggest the potential involvement of miRNAs in regulation of hematopoiesis, T cell activation and differentiation as well as antiviral immunity (12). Abnormal miRNA expression was associated with inflammatory diseases and hematopoietic malignancies (13). The objective of this study was to evaluate the expression of miRNA during human monocyte differentiation into immature DC following stimulation with GM-CSF and IL-4. Here, we identified miR-21 as a potential regulatory small RNA with several target genes involved in DC differentiation.

3. MATERIALS AND METHODS

3.1. Cell cultures

Human peripheral blood mononuclear cells (PBMCs) were prepared by density gradient centrifugation (Lymphoprep,Nycomed Pharm, Oslo, Norway) from buffy coats obtained from healthy adult donors. Cells were washed and then re-suspended in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Monocytes were isolated by plastic adherence. Monocyte-derived immature DC were generated by culturing adherent monocytes in the presence of GM-CSF (25 ng/ml) and IL-4 (50 ng/ml). The cells were harvested at 0 h, 24 h, 48 h and 72 h.

For miR-21 over-expression experiments, isolated monocytes were electroporated with no stimulus or with mature synthetic miR-21 molecules (75 nM) (MGW, Germany) using Human Monocyte Nucleofector kit (Amaxa) according to the manufacturer's recommendations. Cells were seeded into 6-well plates cells were harvested at 24 h post transfection. Total RNA was isolated from cell pellets using Trizol reagent according to the manufacturer's instructions (Invitrogen). The concentration of total RNA was quantified by NanoDrop (Thermo Scientific) and its integrity was evaluated using on Bioanalyzer Nano RNA chip (Agilent).

3.2. Real Time-PCR

Prior RT-PCR, residual genomic DNA was removed by incubating total RNA with 10U of RNase-free DNase I (Roche) for 10 min at 37°C followed by 5 min at 95°C to inactivate the DNase.

3.2.1. miRNA RT-PCR

miRNA qPCR was performed using NCode miRNA SYBR qRT-PCR Kit (Invitrogen) according to the manufacturer's recommendations. Briefly, 500 ng of DNase I-treated total RNA was polyadenylated and cDNA was transcribed using a universal RT primer with SuperScript III RT. The amplification of a specific miRNA performed during a qPCR reaction with SYBR® Green detection reagent priming with the miRNA-specific forward primer and a universal qPCR primer on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each sample was run in duplicate and each PCR experiment included two non-template control wells. The relative expression for each miRNA was estimated from duplicate qPCR reactions following normalization to small nucleolar RNA, C/D box 43 (RNU43) using delta-delta Ct method (14).

3.2.2. mRNA RT-PCR

The cDNA was synthesized from 1 µg DNase I-treated total RNA using the first-strand cDNA synthesis Kit and oligo(dT) primer as recommended by the manufacturer (GE Healthcare) in a 8 µl reaction volume. The cDNA solution was diluted 1:10 with nuclease-free water. Real-time PCR was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and specific primer pairs for the selected genes. Primers were designed using the software Primer Express (Applied Biosystems). For each sample, the following mix was prepared: 2 µl cDNA, 10 µl SYBR Green PCR Master Mix (Applied Biosystems), 300 nM of each primer (Invitrogen) in and nuclease-free water to a final volume of 20 µl. Real-time PCR reactions were run in duplicates according the manufacturer amplification protocol. A final melt curve analysis was included to verify that one specific product was obtained in each reaction. Calculations were based on the delta-delta Ct method (14), as a reference gene, we used human Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.3. Gene expression re-analysis during human monocyte differentiation to DCs

The data set of the study on human monocyte differentiation to DCs driven by GM-CSF and IL-4 *in vitro* that has been published previously (6) was re-analyzed with the following settings: chips were normalized with Robust Microarray Analysis (RMA) in R using Bioconductor, significant differential gene expression levels were detected using oneChannelGUI with the implemented maSigPro package(15) with FDR adjusted p value 0.05, fold change 2.

3.4. Gene expression analysis after miR-21 over-expression

A five hundred ng of total RNA of miR-21 overexpressed and non-transfected samples was applied to

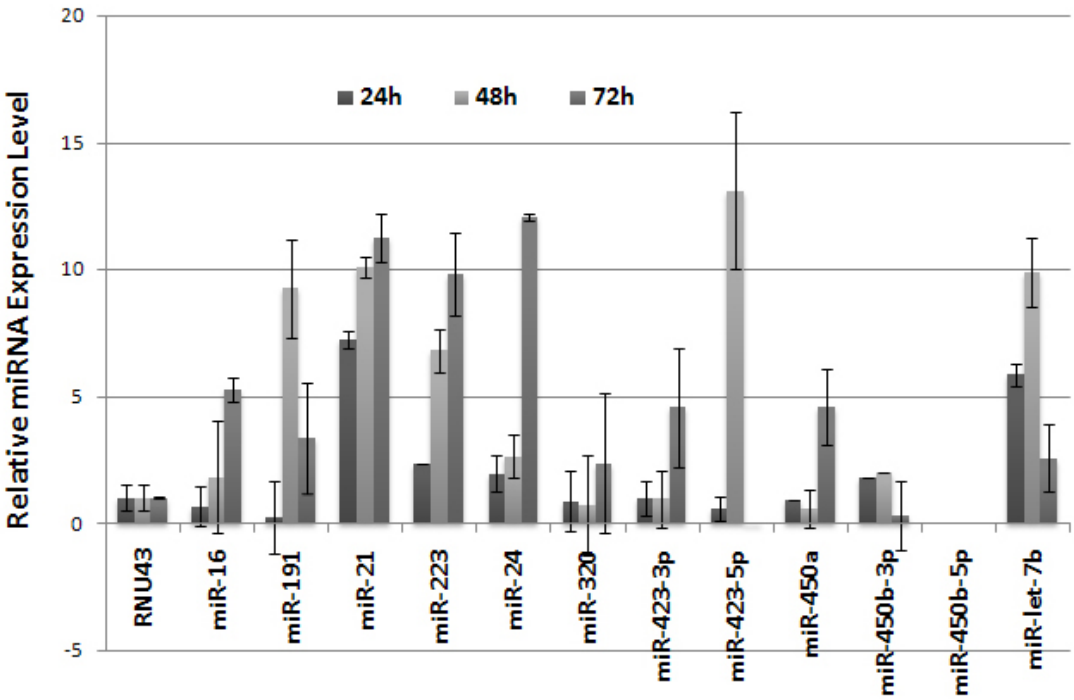

Figure 1. Relative miRNA Expression Level in monocytes v. DCs.The relative quantity of expression for each miRNA was generated from duplicate qPCR reactions following normalization to RNA43. For each miRNA, Ct differences between unstimulated and stimulated at 24 h, 48 h and 72 h were calculated. The fold difference for each miRNA was calculated using delta-delta Ct method.

generate cRNA by using a Illumina TotalPrep RNA Amplification Kit (Ambion). Reverse transcription with the T7 oligo (dT) primer was used to produce first strand cDNA. The cDNA then underwent second strand synthesis and RNA degradation by DNA Polymerase and RNase H, followed by clean up. *In vitro* transcription (IVT) technology, along with biotin UTP, was employed to generate multiple copies of biotinylated cRNA. The labeled cRNA was purified via Filter Cartridge and quantified by NanoDrop (Thermo Scientific) and its integrity was evaluated using on a Bioanalyzer Nano RNA chip (Agilent). The labeled cRNA target (1.5 µg) was used for hybridization to an array according to the Illumina Sentrix humanref-6 beadchip protocol. A maximum of 10 µl cRNA was mixed with a 20 µL GEX-HYB hybridization solution. The preheated 30 µl assay sample was dispensed onto the large sample port of each array and incubated for 18 hours at 58°C at a rocker speed of 5. Following hybridization, the samples were washed according to the protocol and scanned with a BeadArray Reader (Illumina). Expression values were extracted and normalised by the BeadStudio software. Relative fold change was calculated on probes with detection p-value 0.05.

3.5. Computational target prediction, GO and protein interaction analysis

The computationally-predicted target transcripts for miR-21 were obtained using miRBASE Targets (16), TargetScan (17) and Pictar (18) miRNA prediction databases. Functional classification was performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) on-line tools (19). The enrichment of GO processes and KEGG pathways was calculated among the predicted miR-21 target genes using H.sapiens whole genome as background reference. The protein interactions maps were derived from mining interactions from a unique resource that integrates protein interaction terms retrieved from the Biomolecular Interaction Network Database (BIND) (20) with the citations of protein pairs extracted from the same sentence in Medline abstracts. In this resource, the protein citations in Medline are retrieved from the PubGene medical information extraction system (21).

4. RESULTS

4.1. miRNA expression during human monocyte differentiation to DCs

To determine the expression of microRNAs during human peripheral blood monocyte differentiation into DC, monocytes were differentiated *in vitro* to DCs in the presence of differentiation-initiating cytokines, namely GM-CSF and IL-4. Initial analysis of miRNA expression by microarrays showed an alteration of gene expression during cell differentiation. In this study, we selected 12 candidate miRNAs for expression analysis in multiple healthy donors at 24 h, 48 h and 72h post stimulus. MiR-223, miR-16, miR-191, miR-24, let-7b and miR-21 were identified as differentially expressed between monocytes and monocyte derived DCs (Figure 1). We also observed

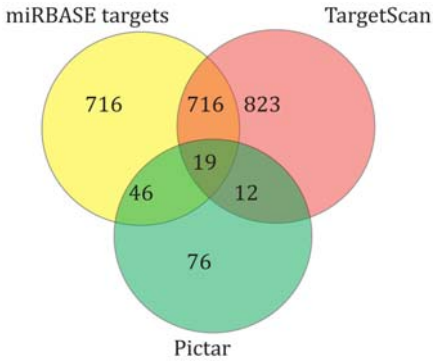

Figure 2. Computationally predicted target transcripts of miR-21. miRanda Targets, TargetScan and Pictar miRNA target prediction databases were used to identify a potential binding sites for miR-21 in genomic sequences. The numbers of computationally-predicted target transcripts are presented in venn diagram. The numbers in the intersection indicates the target genes that were predicted commonly using the indicated databases.

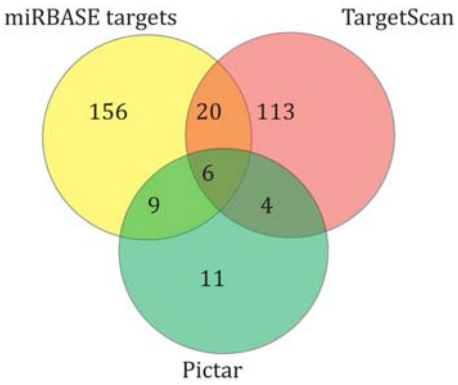

Figure 3. Differentially expressed computationally predicted targets of miR-21 during monocyte differentiation to DCs. miRanda Targets, TargetScan and Pictar miRNA target prediction databases were used to identify a potential binding sites for miR-21 in genomic sequences. The predicted targets futher mapped the differentially expressed genes during monocyte differentiation to DCs. The numbers of computationally-predicted and differentially expressed target transcripts are presented in venn diagram. The numbers in the intersection indicates the target genes that were predicted commonly using the indicated databases.

relatively high variation in expression of analyzed miRNAs across the different donors. However, miR-21, miR-24 and miR-223 represent the most promising candidates because they were upregulated in the most analyzed samples.

4.2. Computationally-predicted mRNA targets of miR-21

Prediction of miRNA target genes provides an alternative approach to assign biological functions. Therefore, we have analyzed miR-21 potential target genes. First, we have used miRNA prediction databases to identify potential binding sites for miR-21 in human genomic sequences. The numbers of computationally-predicted target transcripts for miR-21 using miRBASE Targets (16), TargetScan (17) and Pictar (18) miRNA prediction databases are presented in venn diagram in Figure 2. The number in the intersection indicates commonly predicted targets by the used databases. Secondly, we looked whether miR-21 regulates specific biological processes. The miR-21 target genes that were predicted in at least two databases were used for Gene Ontology (GO) analysis. The enriched biological processes of the predicted targets for miR-21 are presented in Table 1. Interestingly, genes involved in regulation of transcription were among the most significantly enriched, along with genes involved in proteolysis, protein modification and folding in addition to cell cycle control genes. Also, genes involved small molecule transport, receptor protein tyrosine kinase signaling, Jak-STAT signaling and MAPK signaling pathways were enriched for miR-21 targets. Moreover, multiple genes annotated as genes involved in cytokine-cytokine receptor interaction and focal adhesion were among none-significantly enriched miR-21 targets.

4.3. Multiple computationally-predicted miRNA-21 targets were repressed during monocyte differentiation to DCs

The expression profile of human monocyte differentiation to DCs driven by GM-CSF and IL-4 *in vitro* has been published previously (6). The data set of this study was kindly given by authors. We re-analyzed the dataset and mapped the differentially expressed genes to the predicted targets of miR-21. The numbers of differentially expressed genes that have a predicted miR-21 target site are presented in venn diagram in Figure 3. The numbers in the intersections indicate commonly predicted targets by the used databases. Interestingly, several repressed as well as induced genes had a predicted target site for miRNA-21. Among repressed genes cell cycle regulation, such as cyclin G (CCNG1), fos-like antigen 1 (FOSL1), thrombospondin 1 (THBS1), regulator of chromosome condensation (RCC1), phosphodiesterase 4D interacting protein (myomegalin) (PDE4DIP), thiamin pyrophosphokinase 1 (TPK1), stromal antigen 2 (STAG2), pericentriolar material 1 (PCM1) and programmed cell death 4 (neoplastic transformation inhibitor) (PDCD4). Additionally apoptosis regulating genes, such as tumor necrosis factor receptor superfamily, member 10b (TNFRSF10B), BH3 interacting domain death agonist (BID), calpain 2, (m/II) large subunit (CAPN2), phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (PIK3R1), SNF related kinase (SNRK), annexin A1 (ANXA1), annexin A4 (ANXA4) and ras homolog gene family, member B (RHOB) had a predicted biding site for miR-21 and downregulated during the monocyte differentiation to DCs. Multiple genes that encode cell surface receptor mediated signal transduction were modulated. Surprisingly, cAMP responsive element binding protein-like 2 (CREBL2) was upregulated despite having a significant binding site for miR-21. Genes that encode proteins annotated as immunity and defense and MHCI-mediated immunity members were also overrepresented among the genes that were differentially expressed and had a miR-21 target site.

Table 1. Enriched Gene Ontology Categories among miR-21 computationally predicted genes

Term: PANTHER biological process	**Gene Count**	**PValue**
BP00040:mRNA transcription	65	4.45E-04
BP00071:Proteolysis	55	5.48E-04
BP00037:DNA degradation	6	0.001
BP00062:Protein folding	12	0.001
BP00044:mRNA transcription regulation	90	0.001
BP00031:Nucleoside, nucleotide and nucleic acid metabolism	41	0.002
BP00063:Protein modification	34	0.002
BP00248:Mesoderm development	15	0.003
BP00141:Transport	21	0.003
BP00207:Cell cycle control	9	0.003
BP00034:DNA metabolism	14	0.006
BP00145:Small molecule transport	11	0.008
BP00286:Cell structure	36	0.010
BP00289:Other metabolism	32	0.015
BP00250:Muscle development	10	0.024
BP00108:Receptor protein tyrosine kinase signaling pathway	8	0.032
BP00036:DNA repair	16	0.059
BP00114:MAPKKK cascade	5	0.090
Term: KEGG pathway	**Count**	**PValue**
hsa04630:Jak-STAT signaling pathway	6	0.026
hsa04010:MAPK signaling pathway	7	0.066
hsa04060:Cytokine-cytokine receptor interaction	6	0.154
hsa04510:Focal adhesion	5	0.180

4.4. Gene expression profile in response to miR-21 upregulation

In order to validate miR-21 target genes, freshly isolated monocytes were transfected with the mature miR-21 and the cells were harvested after 24 h. The increase of miR-21 levels was confirmed by RT-PCR (Figure 4). To identify transcripts whose levels may be affected by miRNA-21 upregulation, we analyzed mRNA expression profiles in transfected and untransfected monocytes using oligonucleotide microarrays containing probes covering the complete human transcriptome (48,000 transcripts). Totally 1534 transcripts were changed by one fold or more, among them 854 transcripts were downregulated and 680 were upregulated. Since miRNA induced gene expression regulations is known to be fine-tuned, first we looked how many genes among altered genes were highly modulated. As one could expect, only 5.6% of the repressed genes and 3.3% of the induced genes were altered more than 2 fold. Secondly, we used miRNA target prediction databases to identify how many of these predicted targets were differentially expressed in our settings. The analysis showed that only 31 (3.0 %), 51 (5.6 %) and 12 (6.9 %) of the repressed transcripts after overexpression of miR-21 were computationally predicted to have a binding site by miRBASE targets, TargetScan and Pictar miRNA target prediction databases respectively (Table 2). And vice versa, only 3.6 %, 6.0 % and 1.4 % of the repressed transcripts were predicted to have the binding site for miR-21 by miRanda, TargetScan and Pictar (Table 3). There was no correlation between the prediction score and the fold change for the genes predicted.

Interestingly, only DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (DDX17), solute carrier family 16, member 10 (aromatic amino acid transporter) (SLC16A10), STAG2, transcript variant 2, reticulon 4 (RTN4), transcript variant 1 and MYST histone acetyltransferase (monocytic leukemia) 3 (MYST3) were repressed more than two fold among the predicted target genes of miR-21. Member RAS oncogene family (RAB6A), transforming growth factor, beta-induced, 68kDa (TGFBI) and ANXA1, phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila) (PDE4B), CD164 molecule, sialomucin (CD164), CD69 molecule (CD69), CD47 molecule (CD47), TGFBI and PDCD4 were among other moderately repressed while TIAM1 was induced. Interestingly Dicer1, Dcr-1 homolog (Drosophila) (DICER1), transcript variant 2, was also predicted target for miR-21 and was moderately repressed. A relatively small number of genes predicted to have a miR-21 target site were also moderately (less than 2 fold) induced. Although further studies are required, gene induction could result from the downregulation of miR-21 target genes.

For a gene to be regulated by miRNA, it must be expressed in the cell. Therefore, we looked for a potential association between the genes that are expressed in monocytes and miR-21 dowregulated genes. Interestingly, the predicted target genes that were experimentally identified as repressed transcripts, were also highly expressed in monocytes. While approximately 50% of the predicted target genes that were not validated experimentally were expressed at medium or low levels. However, there was no direct correlation between the expression levels and the differential expression of miR 21 target genes. Furthermore, the target genes that were induced by miR-21 upregulation in monocytes were also highly expressed.

4.5. Cross validation of the selected genes

Taking into account that miR-21 was induced in monocytes during the differentiation to DCs, one may assume that the expression miR-21 in monocytes will target analogous genes. Therefore, we mapped the genes that had a predicted miR-21 target site and differentially expressed in monocytes during the differentiation to DCs to the modulated genes after miR-21 transfection in monocytes. A moderate number (39 from 102) of genes were present in both lists (Figure 5). However, there was no direct correlation in expression levels between the genes that are

Table 2. Mapping of computationally predicted miR-21 targets to the differentially expressed genes

	Totally predicted	dif.expr.pr	repressed	induced
Mirbase targets	1040	44(4.2%)	31(3.0%)	14(1.3%)
TargetScan	909	65(7.2%)	51(5.6%)	14(1.5%)
Pictar	175	15(8.6%)	12(6.9%)	3(1.7%)

% of predicted genes that were differentially expressed

Table 3. Mapping of computationally predicted miR-21 targets to the expression data

	Mirbase	TargetScan	Pictar
	1040	909	175
dif.expr.pr	44(2.9%)	65(4.2%)	15(1.0%)
repressed	31(3.6%)	51(6.0%)	12(1.4%)
induced	14(2.1%)	14(2.1%)	3(0.4%)

% of differentially expressed genes that were predicted to have binding site

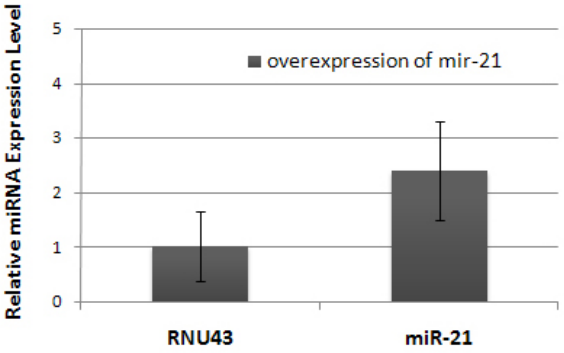

Figure 4. Relative Expression Level of miR-21 in monocytes after over-expression. The relative quantity of expression for miR-21 was measured from duplicate qPCR reactions following normalization to RNA43. The bars show the calculated Ct differences between untransfected and transfected with miR-21 at 24 h post transfection.

expressed during monocyte differentiation and the genes expressed in monocytes following to miR-21 upregulation. As expected multiple genes were repressed in both datasets, among them PDE4B, TGFBI, PDCD4, ANXA1, STAG2, s100 calcium binding protein a12 (calgranulin c) (S100A12), CD69 molecule (CD69) and nuclear receptor interacting protein 1 (NRIP1). The analysis mRNA expression of TGFBI, PDCD4, ANXA1 following over-expression of miR-21 by RT-PCR verified the microarray data (Figure 6). However, the expression of several genes was different in the datasets, indicating the potential false positives or negatives targets.

4.6. Protein interaction maps of the identified targets

Protein interaction maps provide a valuable framework for a broader understanding of the functional interconnections of the target genes and their affected processes. To detect interacting pairs of the identified miR-21 targets, a matrix of interacting protein-protein pairs for each target was created. Additionally, GO process enrichment of the genes in the maps was estimated for each map. Interestingly, multiple enriched GO processes of the miR-21 target maps overlapped, such as cell cycle, death and differentiation, gene expression and regulation of transcription, signal transduction and transport (Table 4).

Interestingly, only PDE4B has the predicted binding site for miR-21, however the interacting protein-protein pairs of PDE4B identified also other phosphodiesterases 4A, 4B, 4C, 4D and 3A and 3B were also repressed during monocyte differentiation to DCs (Figure 7). STAG2 and rad21homolog (s. pombe) (RAD21) are components of cohesin complex, a complex required for the cohesion of sister chromatids after DNA replication. Both STAG2 and RAD21 are predicted miR-21 targets, RAD21 is also downregulated in during the monocyte differentiation to DCs, however the multiple testing analysis did not find the gene significant and therefore it was not detected by previous analysis. Interestingly, other important cell cycle regulators were also repressed such as aurora kinase b (AURKB), polo-like kinase 1 (drosophila) (PLK1), rec8-like 1 (yeast) (REC8) and smc1 structural maintenance of chromosomes 1-like 1 (yeast) (SMC1A) (Figure 7). Protein interacting pair genes of NRIP1, ANXA1, PDCD4 and ING3 maps grouped into pro-apoptotic genes. LAT2 and S100A12 protein-protein interacting pairs were associated with transport, while TGFBI related to cytosckeletal components and may modulate cell-collagen interactions and cell adhesion.

5. DISCUSSION

Hematopoietic lineage differentiation is known to be controlled by complex molecular events that simultaneously regulate the commitment, proliferation, apoptosis, and maturation of hematopoietic stem/progenitor cells. Current estimates suggest that about one-third of human mRNAs are regulated by miRNAs (22). The picture gets even more complicated when one single miRNA may have several mRNA targets and several miRNAs may regulate one single target mRNA. Additionally, the same miRNAs might regulate different mRNA transcripts in the different cell types. In this study, we identified that miR-223, miR-16, miR-191, miR-24, let-7b and miR-21 were upregulated in monocytes in response to GM-CSF and IL4.

Target mRNAs are recognized by miRNAs through sequence complementarity, usually between the miRNA and sequences in the 3-UTR of the mRNA. miRNAs reported to silence target genes either through inhibition of initiation of translation, which results in prevention of ribosome association with the target mRNA, or through inhibition of translation post-initiation, including promoting deadenylation, which might result in

Table 4. Enrichment of GO processes in the miR-21 target gene maps

	PDE4B	S100A12	STAG2	TGFBI	PDCD4	NRIP1	LAT2	ING3	ANXA1
GO:0007049 cell cycle			9		5			9	
GO:0008219 cell death	6				4	4		5	7
GO:0030154 cell differentiation	10	5			7	5		6	7
GO:0010467 gene expression					11	11			
GO:0006355 regulation of transcription						11		11	
GO:0007165 signal transduction	12			12		9			
GO:0007242 transport		5					12		

*numbers indicates gene counts in the enriched GO processes in the specified gene maps

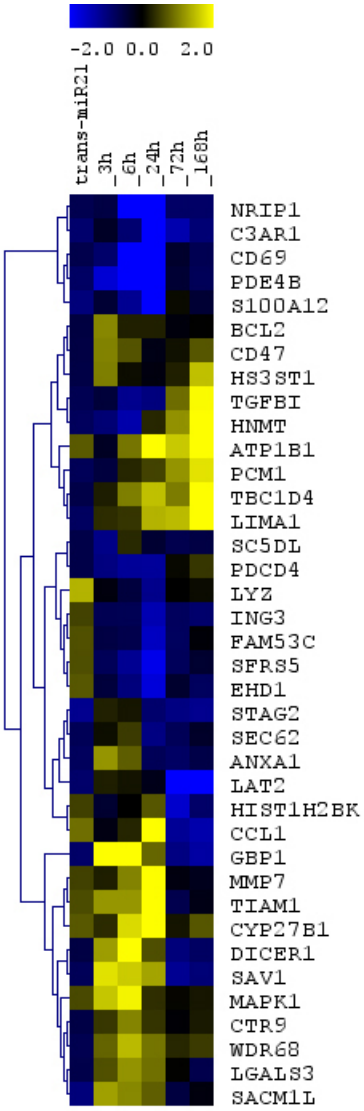

Figure 5. The expression profile of computationally predicted targets of miR-21 that were differentially expressed during monocyte differentiation to DCs and over-expression of miR-21. A cluster of computationally predicted targets of miR-21 that were differentially expressed during monocyte differentiation to DCs and over-expression of miR-21. Horizontal stripes represent genes and columns show treatment protocols.

degradation of mRNA (8). The fact that miRNAs can cause degradation of their targets facilitated the use of microarray technology to identify miR-21 target genes in monocytes differentiation to DCs. Of note, recent studies showed that human miRNAs can alter mRNA stability, leading to mRNA degradation that can be detected by transcriptional microarrays (23).

Assessment of miRNA target genes provides an alternative approach to assign biological function to miRNAs. Three

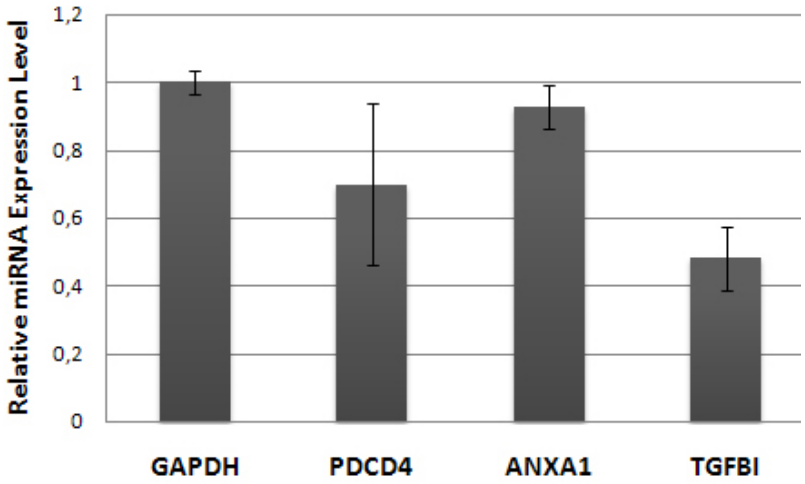

Figure 6. Relative Expression Level selected miR-21 predicted targets in monocytes after over-expression. The relative quantity of expression for each gene was generated from duplicate qPCR reactions following normalization to GAPDH. The bars show the calculated Ct differences for the indicated targets between untransfected and transfected with miR-21 at 24 h post transfection.

miRNA prediction databases were used to identify potential binding sites for miR-21 in human genomic sequences. Only a small number of predicted miRNA targets have been validated so far. The correlation of the predicted target genes to differentially expressed genes during monocyte differentiation to DCs showed comparative successes rate for all three prediction databases. Over-expression of miR-21 repressed the expression of a set of genes some of that were also repressed during monocyte differentiation to DCs.

The identified potential miR-21 targets were linked to cell cycle, death and differentiation. Both STAG2 and RAD2 were among the identified potential miR-21 targets and components of cohesin complex that forms a large proteinaceous ring within which sister chromatids can be trapped. At anaphase, the complex is cleaved and dissociates from chromatin, allowing sister chromatids to segregate. Furthermore, other important cell cycle regulators were also repressed. Cell cycle blockade likely is necessary to proceed through the differentiation. Moreover, miR-21 targeted multiple key pro-apoptotic genes. NRIP1 is a crucial adaptor kinase that is induced by stress signaling pathways and promotes apoptosis and activation of NF-kappa-B. While ANXA1 is a 37 kDa member of the annexin superfamily that structurally belongs to a family of ubiquitous phospholipids and calcium-binding proteins. Depending on the cell system, it is implicated in apoptosis induction, caspase-3 activation, cell growth inhibition, and phagocytosis. PDCD4 gene expression was found to be upregulated during apoptosis induced by various stimuli, although the mechanism by which it affects apoptosis is not known (24). PDCD4 is the most validated miR-21 target (25-29). ING3 is required for the activation of transcriptional programs associated with apoptosis (30). Given that, the miR-21-mediated downregulation of multiple pro-apoptotic genes might be essential for the monocyte differentiation to DCs.

Additionally gene expression and regulation of transcription, signal transduction and transport networks were revealed. Phosphodiesterases (PDE) are an important cAMP-metabolising enzymes in immune and inflammatory cells that plays a significant role in modulating the activity of cAMP, an important second messenger that mediates the relaxation of airway smooth muscle and suppresses inflammatory cell function, thereby attenuating the inflammatory response. Selective inhibitors of PDE4enzyme show a broad spectrum of activity in animal models of chronic obstructive pulmonary disease and asthma. These drugs block the hydrolysis of cAMP via inhibition of PDE4 and are attractive candidates for novel anti-inflammatory drugs (31). PDE4B identified as potential target of miR-21. It is tempting to speculate that antimiR-21 could be used as PDE4B inhibitor in inflammatory diseases.

Other previously validated miR-21 target genes such as tropomyosin 1 (alpha) (TPM1), Fas (TNF receptor superfamily, member 6) (FAS), serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5), nuclear factor I/B (NFIB) were not expressed or expressed at low levels in monocytes. Despite the fact that cyclin-dependent kinase 6 (CDK6), phosphatase and tensin homolog (mutated in multiple advanced cancers 1) (PTEN), apoptotic peptidase activating factor 1 (APAF1), interleukin 6 receptor (IL6R),sprouty homolog 1, and antagonist of FGF signaling (Drosophila) (SPRY1) transcripts were moderate or highly expressed in monocytes, these previously validated targets were not altered by miR-21 upregulation in monocytes. This observation underlies the complexity of miRNA target gene validation and indicates that miRNA can discriminate between target genes. Therefore, it is important to address the roles that direct miRNA specificity in different tissues and cell types to best identify a target gene and its function.

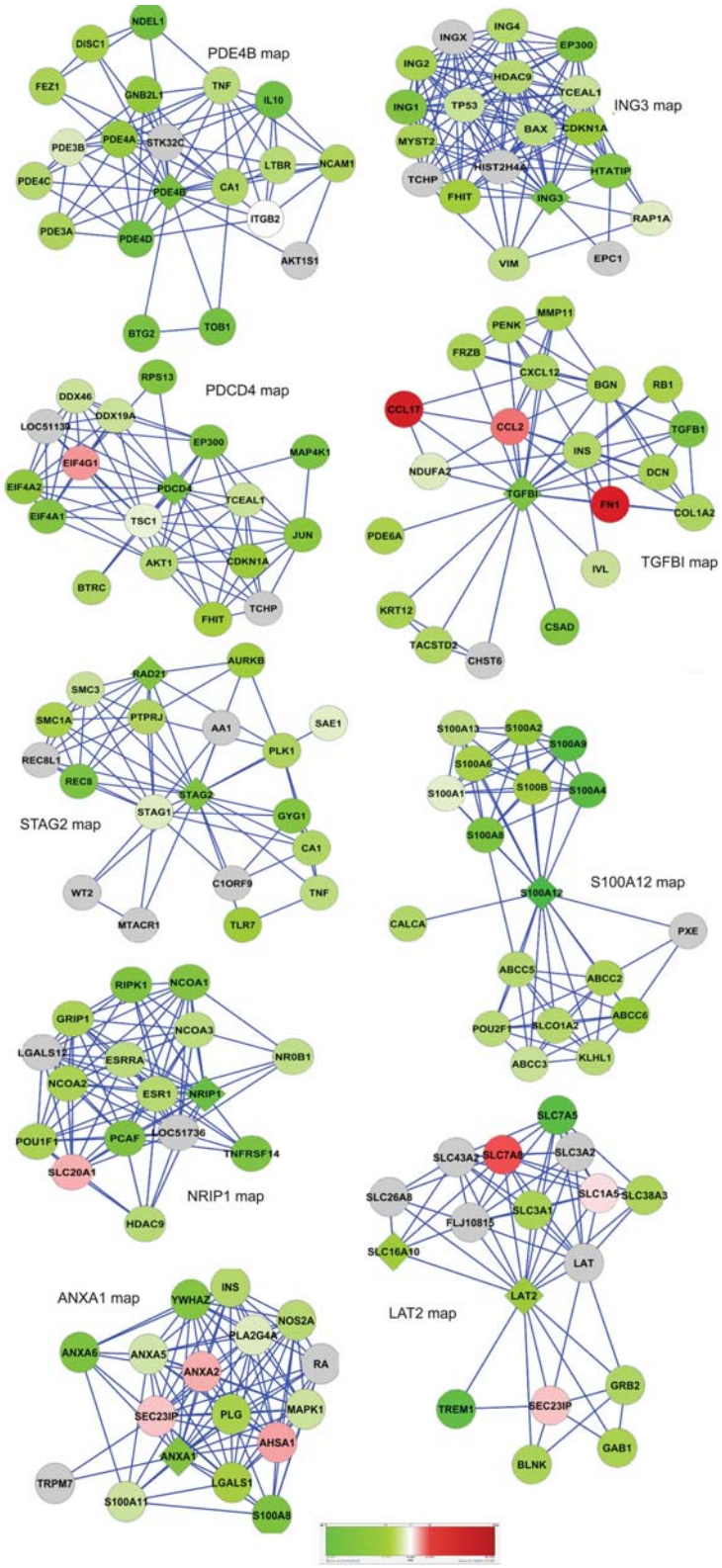

Figure 7. Protein interaction maps of the identified targets. The square nodes in the network clusters are the target genes and the circular nodes are those genes that potentially interact with the target gene in the human interactome. The genes are color coded according to their expression levels, where the degree of color intensity for green represents down-regulation and for red represents up-regulation. Grey genes were not present on microarrays.

During the preparation of this paper, Hashimi and colleagues reported on the induction of miR-21 and miR-34a expression in monocyte differentiation to DCs (32). The authors identified wingless-type MMTV integration site family, member 1 (WNT1) and jagged 1 (Alagille syndrome) (JAG1) as a target genes for miR-21. However, we did not observe changes in WNT1 and JAG1 gene expression after miR-21 transfection in monocytes. JAG1 showed reduced expression during the early monocyte differentiation (6h) to DCs followed by increase in expression at the late differentiation stages (72 h and 168 h). Similarly, no significant changes in WNT1 mRNA levels were detected during monocytes differentiation to DC. Discrepancy of these results maybe because of these genes might not be susceptible to degradation and therefore may not have been identified by mRNA microarray profiling. However, our results and the recent studies of global analysis of mRNA and protein levels indicated that miRNA induced gene silencing is frequently mirrored by decreased transcript levels (33, 34), suggesting that potential miRNA targets can be identified without a need for sophisticated proteomics approaches.

Collectively, our data suggest that miR-21 is a key regulatory miRNA induced during monocyte differentiation to DC. MiR-21 repressed a certain set of genes during monocytes differentiation to DCs that were also repressed by miR-21 transfection in monocytes. The identified potential miR-21 targets take role in cell cycle regulation, repression of the pre-apoptotic genes and regulation signal transduction during the DCs differentiation process.

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Abbreviations: miR: microRNA, DCs: dendritic cells, GO: Gene Ontology

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