Structural evidence of anti-atherogenic microRNAs

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1. ABSTRACT

Our research attempted to address two important questions - how microRNAs modulate atherogenic inflammatory genes from a panoramic viewpoint and whether their augmented expression results from reduced microRNAs suppression. To resolve these knowledge gaps, we employed a novel database mining technique in conjunction with statistical analysis criteria established from experimentally verified microRNAs. We found that the expression of 33 inflammatory genes up-regulated in atherosclerotic lesions contain structural features in the 3'UTR of their mRNAs for potential microRNAs regulation. Additionally, the binding features governing the interactions between the microRNAs and the inflammatory gene mRNA were statistically identical to the features of experimentally verified microRNAs. Furthermore, 21 of the 33 inflammatory genes (64%) were targeted by highly expressed microRNAs and 10 of these (48%) were targeted by a single microRNA, suggesting microRNA regulation specificity. Supplementing our findings, 7 out of the 20 unique microRNAs (35%) were previously confirmed to be down-regulated when treated with pro-atherogenic factors. These results indicate a critical role of anti-inflammatory microRNAs in suppressing pro-atherogenic inflammatory gene expression.

2. INTRODUCTION

Cardiovascular disease (CVD) has been researched for decades leading to a long held understanding and strong characterization of the traditional and non-traditional risk factors. Despite this, the mechanism of onset has only recently been elucidated. As a chronic inflammatory disease, atherosclerosis development is dependent on the activity of both the adaptive and innate immune systems (1). For example, we and others have reported that $CD4^+CD25^{high}$ regulatory T cells (2-4), an adaptive immune cell, and $Ly6C^{mid/high}$ monocytes, an innate immune cell, both play integral roles in modulating atherogenesis and vascular inflammation (5). In addition, it is accepted that the activation, inflammation, and dysfunction of endothelial cells are responsible for the initiation of atherosclerosis (6). Once activated, endothelial and vascular smooth muscle cells secrete pro-inflammatory cytokines and chemokines which attract monocytes and T cells (7). This leads to further endothelium and vessel inflammation causing plaque build-up and lesion formation over time. However, it remains unclear how detailed gene regulation mechanisms modulate this process.

Recent publications suggest that microRNAs (miRNAs), a newly characterized class of short (18-24

nucleotide long) (8), endogenous, non-coding RNAs, contribute to the development of particular disease states through the regulation of diverse biological processes such as cell growth, differentiation, proliferation, and apoptosis (9). This biological control is accomplished by post-transcriptional gene silencing (10) through Watson and Crick base-pairing predominately at the 3'-untranslated region (3'UTR) of messenger RNAs (mRNAs) (11, 12). This pairing can be further characterized as "perfect" or "near perfect", leading to target mRNA cleavage and degradation, or "imperfect", causing the inhibition of mRNA translation (10). With the identification and sequencing of more than 800 human miRNAs thus far, it is thought that up to 30% of human genes may be regulated by miRNAs (9, 13). Supporting evidence suggests that miRNAs function as key players during critical stages of cellular development and finely tune gene expression in the maintenance of routine cellular functioning (14). Furthermore, miRNAs can act on transcription factors, which leads to a broad indirect cellular effect as a result of the widespread gene modulating nature of transcription factors. The ability of individual miRNAs to exert control over the expression of an array of genes provides insight into how miRNAs deregulation can contribute to disease development.

The involvement of miRNAs in the maintenance of healthy homeostasis, inflammation, and their expression in vascular tissue has been examined independently in the literature (15, 16). However, integration of these disciplines to investigate the potential miRNAs suppression of pro-atherogenic inflammatory genes still remains to be conducted. To address this short-coming, we examined the role of miRNAs in the inhibition of pro-inflammatory genes differentially expressed in atherosclerotic lesions. Utilizing database mining techniques and statistical analysis from a panoramic viewpoint, we generated a list of miRNAs which are believed to suppress inflammation development necessary for atherosclerosis progression. In-depth observation of these miRNAs could prove vital in further understanding the underlying suppressive mechanism for atherosclerosis onset and progression. Such knowledge may unveil novel avenues for innovative therapeutic treatments like miRNA mimics and inhibitors (17) for atherosclerosis and other cardiovascular diseases.

3. MATERIALS AND METHODS

3.1. Compilation of an inflammatory gene list that is modulated in atherosclerotic lesions

A database mining approach was employed to identify inflammatory genes and miRNAs which are potentially involved in atherosclerosis (Figure 1). A list of 101 genes whose expression levels were experimentally determined to be modulated in atherosclerotic lesions as published by Tabibiazar et al. in 2005 was generated (18). The list of 101 genes was further narrowed based on gene involvement in various aspects of the inflammatory process. This was accomplished by cross-referencing/matching the first list of 101 genes with a second list containing all genes predetermined to contribute to inflammatory development published by Loza et al. (19).

3.2. Prediction of miRNAs which potentially target the list of pro-atherogenic inflammatory genes

Potential miRNAs targets were examined with the online miRNAs target prediction software, TargetScan (http://www.targetscan.org/) (20). Both the context value and percentage were employed to gauge binding relevance. The context value delineates the interaction's "goodness of fit" while the context percentage describes the number of predicted binding sites with a lower context value for a particular miRNA. Each gene was examined and both the "conserved" and "poorly conserved" miRNAs binding sites were recorded along with their context values and percentages (21).

3.3. Expression of miRNAs within vascular tissues and/or inflammatory cells

Tissue and cell-specific miRNA expression was examined with the online microRNA.org expression database (http://www.microrna.org/microrna/home.do) (22). The methodology utilized to normalize miRNAs' expression in vascular tissues/inflammatory cells was described previously (22).

3.4. Statistical analysis

Statistical analyses were performed using the functions of t test, confidence intervals and the Pearson's chi square test in Microsoft Office Excel (23).

4. RESULTS

4.1. Generation of a list of 33 inflammatory genes modulated in atherosclerotic lesions

We hypothesized that the inflammatory genes up-regulated in atherosclerotic lesions could be the result of reduced miRNAs interactions. To test this theory, we developed an innovative database mining strategy which required the identification of inflammatory genes whose expressions were experimentally determined to be up-regulated in atherosclerotic lesions (Figure 1). Previously published by Tabibiazar et al., we utilized gene expression microarray data from atherogenic apolipoprotein E deficient (ApoE$^{-/-}$) mice and human atherosclerotic coronary artery samples to develop a list of 101 human genes (24). This list was then further examined for involvement in the inflammatory process. To accomplish this, a second list with all genes predetermined to contribute to inflammatory development, published by Loza et al., was used to cross-reference the first list of 101 genes (19). Upon filtration, 33 human inflammatory genes remained. The expressions of these genes were up-regulated in atherosclerotic lesions in a range of 1.19 to 4.29 fold (Table 1). These 33 genes were found to participate in a variety of inflammation aspects, including five genes in adhesion and migration, one gene in calcium signaling, three genes in the complement cascade, seven genes in cytokine signaling, two genes in eicosanoid signaling, two genes in G-protein coupled receptor signaling, six genes in leukocyte signaling, three genes in the mitogen activated protein kinase (MAPK) pathway, two genes in tumor necrosis factor receptor signaling, one gene in antigen presentation, and one in TNF signaling (Table 1).

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Table 1. Thirty-three genes were modulated in atherosclerotic plaques and found to participate in inflammation

Gene	NCBI ID #	Fold change	Primary Pathway
CXCL16	**58191**	**2.01**	**Adhesion-Extravasation-Migration**
FYB	**2533**	**1.47**	**Adhesion-Extravasation-Migration**
ITGA6	**3655**	**1.75**	**Adhesion-Extravasation-Migration**
RHOH	**399**	**1.76**	**Adhesion-Extravasation-Migration**
VCAM1	**7412**	**1.82**	**Adhesion-Extravasation-Migration**
PPP3CA	5530	1.52	Calcium Signaling
C1QA	712	2.55	Complement Cascade
C1QB	**713**	**1.37**	**Complement Cascade**
C1R	**715**	**2.01**	**Complement Cascade**
CASP1	834	1.67	Cytokine signaling
CSF1	**1435**	**1.50**	**Cytokine signaling**
CSF1R	**1436**	**1.59**	**Cytokine signaling**
IL10RA	**3587**	**2.33**	**Cytokine signaling**
IRF8	**3394**	**1.64**	**Cytokine signaling**
PTPN2	5771	1.45	Cytokine signaling
TGFBR2	**7048**	**1.69**	**Cytokine signaling**
ALOX5AP	241	1.42	Eicosanoid Signaling
LTA4H	4048	1.48	Eicosanoid Signaling
PDE1A	**5136**	**1.44**	**G-Protein Coupled Receptor Signaling**
RGS1	**5996**	**4.29**	**G-Protein Coupled Receptor Signaling**
CD53	963	2.35	Leukocyte signaling
FCER1G	2207	1.49	Leukocyte signaling
FCGR2B	**2213**	**2.39**	**Leukocyte signaling**
LCP2	**3937**	**1.66**	**Leukocyte signaling**
PTPRC	**5788**	**2.03**	**Leukocyte signaling**
SYK	6850	1.63	Leukocyte signaling
LYN	**4067**	**1.97**	**MAPK signaling**
MKNK1	**8569**	**1.24**	**MAPK signaling**
RAC2	5880	2.45	MAPK signaling
NFKB1	396033	1.26	NF-kB signaling
NFKBIA	4792	1.55	NF-kB signaling
TAP1	**6890**	**1.19**	**Phagocytosis-Ag presentation**
TNFRSF1A	**7132**	**1.24**	**TNF Superfamily Signaling**

Bolding denotes genes which contain predicted miRNA binding sites of interest. CXCL16 - Chemokine ligand 16; FYB – FYN binding protein; ITGA6 – Integrin alpha 6; RHOH – Ras homolog gene family, member H; VCAM1 – Vascular cell adhesion molecule 1; PPP3CA – Protein phosphatase 3, catalytic subunit, alpha isozyme; C1QA – Complement component , 1 q subcomponent, A chain; C1QB - Complement component, 1 q subcomponent, B chain; C1R - Complement component 1, r subcomponent; CASP1 – Caspase 1; CSF1 - Colony stimulating factor; CSF1R - Colony stimulating factor 1 receptor; IL10RA - Interleukin 10 receptor, alpha; IRF8 - Interferon regulatory factor 8; PTPN2 – Protein tyrosine phosphatase, non-receptor type 2; TGFBR2 - Transforming growth factor, beta receptor II; ALOX5AP - Arachidonate 5-lipoxygenase-activating protein; LTA4H - Leukotriene A4 hydrolase; PDE1A - Phosphodiesterase 1A, calmodulin-dependent; RGS1 - Regulator of G-protein signaling 1; CD53 - CD53 molecule; FCER1G - Fc fragment of IgE, high affinity I, receptor for gamma polypeptide; FCGR2B - Fc fragment of IgG, low affinity IIb, receptor; LCP2 - Lymphocyte cytosolic protein 2; PTPRC - Protein tyrosine phosphatase, receptor type, C; SYK - Spleen tyrosine kinase; LYN – V-yes-1 Yamaguchi sarcoma viral related oncogene homolog; MKNK1 - MAP kinase interacting serine/threonine kinase 1; RAC2 - Ras-related C3 botulinum toxin substrate 2; NFKB1 - Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; NFKBIA - Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; TAP1 - Transporter 1, ATP-binding cassette, sub-family B; TNFRSFIA - Transporter 1, ATP-binding cassette, sub-family B

4.2. Atherosclerotic inflammatory genes have structural features in the 3'UTR of their mRNAs receptive to potential miRNAs regulation

After the identification of the 33 atherosclerosis up-regulated inflammatory genes we postulated that structural features within the 3'UTR of their mRNAs could potentially serve as targets for miRNAs regulation. To test this, we used the online miRNA target prediction software TargetScan (http://www.targetscan.org/) developed by the Bartel Lab from the Whitehead Institute, MIT. This software was selected due to its inclusion of "conserved" and "poorly conserved" miRNAs binding sites, the individual ranking of target miRNA/mRNA binding efficacy, and the wide use of this target prediction program (17, 25, 26). The interaction of miRNAs with target mRNAs is primarily mediated by nucleotides 2-7 in the 5'region of the miRNA, commonly referred to as the

"miRNA seed".   It should be noted that the other nucleotides of the miRNAs probably have some modifying effect as well (8). The individually ranked miRNA binding efficacy assessed by TargetScan was denoted in a context value, which allowed us to analyze the quantitative data with statistical tools. This value was generated using four criteria found to effect miRNA binding efficacy - 3' UTR binding site location, AU sequence richness, seed matching, and additional pairing outside the seed region (20). Additionally, context percentage was employed to gauge binding relevance. This percentage delineates the number of predicted binding sites for a particular miRNA with a lower context value. The mRNAs of each of the 33 genes were examined, and both the "conserved" and "poorly conserved" miRNAs binding sites were recorded along with their context values and percentages (21). This analysis in TargetScan yielded 524 miRNAs, which were
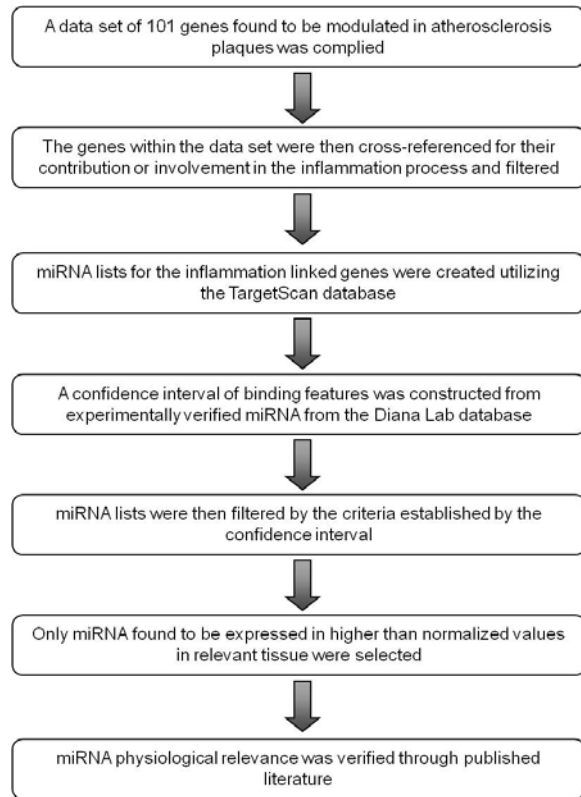
Figure 1. Database mining analysis flowchart with regard to predicted miRNAs binding of inflammatory genes found to be up-regulated in atherosclerotic lesions.

predicted to participate in 1368 unique interactions with the 33 inflammatory gene mRNAs. To ensure relevance, we examined the context value and percentage of experimentally verified miRNAs. Confidence intervals were generated from 45 interactions between 28 experimentally verified human miRNAs and 36 genes found within Tarbase, an online database of experimentally verified miRNAs http://diana.cslab.ece.ntua.gr/tarbase/) (27, 28). These experimental interactions were also selected based on their confirmation by luciferase reporter assays and single site specificity. The 45 miRNA/mRNA interactions that met these criteria were then evaluated in TargetScan to determine the miRNA context values and percentages (Table 2). Analysis of this data yielded a mean and standard deviation (SD) of -0.25 ± 0.12 and 76.07 ± 19.07 for context value and context percentage, respectively. The intervals were then constructed and the lower limits (the mean - 2 x standard deviations) were calculated for context percentage (76.07-1.96 (19.07/SQRT(46)) = 76.07 - 5.51 = 70.56) and context value (-0.25-1.96(0.12/SQRT(46) = - 0.25 - 0.04= -0.22). All predicted miRNAs interactions with a context value ≤-0.22 and context percentage ≥70 were accepted. Using the lower limit thresholds for context value and percentage, 297 out of the 524 predicted miRNAs met the criteria and were considered equivalent to the experimentally verified miRNAs. These results suggest that atherosclerotic inflammatory genes have structural features in the 3'UTR

of their mRNAs which are potentially regulated by miRNAs, and that these features are statistically identical or equivalent to experimentally verified miRNAs.

4.3. 21 out of 33 atherosclerotic inflammatory genes (64%) were targeted by highly expressed miRNAs

Since miRNAs can have differential tissue expression patterns, we hypothesized that atherosclerosis up-regulated inflammatory genes would be regulated by miRNAs that have high expression levels in atherosclerosis related tissues and cells under normal untreated conditions. Tissue and cell-specific miRNA expression was examined to test this theory with the online microRNA.org expression database (http://www.microrna.org/microrna/home.do) (22). The miRNAs with greater than normalized expression levels within appropriate tissues were recorded. The 25 miRNAs with elevated expression in adaptive immune cells (B lymphocytes and T lymphocytes), innate immune cells (dendritic cells, monocytes and splenocytes), and cardiovascular tissues, were recorded in Table 3A. Four types of tissue/cell expression patterns were discovered upon analysis (Table 3B). One of the 25 miRNAs (4%), miR-16, was ubiquitously expressed in every tissue/cell analyzed while 9 out of the 25 miRNAs (36%) were expressed in multiple tissues/cells, including miR-29a, miR-29b, miR-150, miR-15a, miR-27a, miR-143, miR-30d, miR-26a, and miR-30e. Furthermore, 15 out of the 25 miRNAs (60%) were expressed in a single cell type or tissue including miR-29c, miR-1, miR-27b, miR-451, miR-92a, miR-181a, miR-22, miR-223, miR-7, miR-141, miR-32, miR-374a, miR-30c, miR-140-5p, and miR-19b. We considered these miRNAs to be cell-specific or tissue-specific. Moreover, 5 out of the 25 miRNAs (20%) were only expressed in certain functional/activation cell type states, including miR-32, miR-374a, miR-30c, miR-140-5p, and miR-19b. For example, miR-30c was expressed in naïve CD4$^+$ T cells but not in effector CD4$^+$ T cells nor in memory CD4$^+$ T cells. In contrast, miR-32 and miR-374a were expressed in effector CD4$^+$ T cells but not in naïve CD4$^+$ T cells nor in memory CD4$^+$ T cells.

The list of miRNAs with elevated expression levels (Table 3A) was then used to further filter the miRNAs predicted to target atherosclerosis up-regulated inflammatory genes. The results showed that the 25 highly expressed miRNAs targeted 21 out of the 33 inflammatory genes (64%). Of note, these 21 inflammatory genes can be targeted by both highly expressed miRNAs and normally expressed miRNAs. Meanwhile, the other 12 inflammatory genes (36%) were found only to be targeted by normally expressed miRNAs. Thus, the 272 normally expressed miRNAs can target all of the 33 inflammatory genes. As shown in Table 4, 10 of the 21 highly expressed miRNA targeted inflammatory genes (48%) were targeted by a single miRNA while 11 out of 21 inflammatory genes (52%) were targeted by multiple miRNAs. These results indicate that almost half of inflammatory genes are regulated by a single miRNA. In addition, 12 out of 25 miRNAs (48%) targeted single inflammatory genes, whereas the remaining 13 miRNAs targeted multiple inflammatory genes.

Table 2. The context value and % of experimentally verified miRNA found in the Diana Lab database were used to construct a confidence interval to ensure predicted miRNA significance

Gene	miRNA	Context Value	Context %	Conservation
AGTR1	miR-155	-0.19	58	Poorly Conserved
BACE1	miR-9	-0.28	88	Conserved
BCL2	miR-15b	-0.11	44	Conserved
BCL2	miR-16	-0.11	47	Conserved
C1QBP	miR-375	-0.09	39	Conserved
CCND1	miR-34a	-0.11	48	Conserved
CDKN1C	miR-222	-0.19	68	Conserved
CDKN1C	miR-221	-0.19	68	Conserved
COL15A1	miR-29c	-0.44	97	Conserved
COL4A2	miR-29c	-0.3	89	Conserved
CYP1B1	miR-27b	-0.21	74	Conserved
DNMT3A	miR-29a	-0.28	86	Conserved
DNMT3A	miR-29b	-0.28	86	Conserved
DNMT3A	miR-29c	-0.28	86	Conserved
DNMT3B	miR-29a	-0.4	96	Conserved
DNMT3B	miR-29b	-0.4	96	Conserved
DNMT3B	miR-29c	-0.4	96	Conserved
E2F3	miR-34a	-0.44	99	Conserved
EFNA3	miR-210	-0.18	79	Conserved
EZH2	miR-26a	-0.41	94	Conserved
GRIA2	miR-181b	-0.18	53	Conserved
HAND2	miR-1	-0.2	74	Conserved
HOXA5	miR-130a	-0.18	56	Conserved
HOXD10	miR-10b	-0.21	87	Conserved
KCNJ2	miR-1	-0.23	80	Conserved
LAMC1	miR-29c	-0.21	73	Conserved
LIN28	let-7b	-0.32	93	Conserved
LIN28	miR-125b	-0.27	91	Conserved
MCL1	miR-29b	-0.2	71	Conserved
MEOX2	miR-130a	-0.26	79	Conserved
MTPN	let-7b	-0.1	41	Conserved
MTPN	miR-124	-0.1	32	Conserved
MTPN	miR-375	-0.14	71	Conserved
NFIA	miR-223	-0.4	95	Conserved
PDCD4	miR-21	-0.43	96	Conserved
RB1	miR-106a	-0.14	52	Conserved
TAC1	miR-130a	-0.18	59	Poorly Conserved
TERT	miR-138	-0.27	89	Poorly Conserved
TMSL1	miR-1	-0.5	99	Conserved
TP53INP1	miR-155	-0.39	92	Conserved
TPPP3	miR-16	-0.24	83	Poorly Conserved
USP1	miR-375	-0.13	64	Poorly Conserved
UTRN	miR206	-0.3	90	Conserved
ZBTB10	miR-27a	-0.18	66	Conserved
ZEB2	miR-192	-0.51	99	Conserved

4.4. The miRNAs targeting atherosclerotic inflammatory genes use statistically higher numbers of "poorly conserved" binding sites than a control group of miRNAs

Due to the pathological driven expression modulation of atherosclerosis up-regulated inflammatory genes, we hypothesized that the miRNA/mRNAs interactions were distinct in some fashion when compared to interactions involved in standard physiological conditions. There were 42 unique miRNA/mRNA interactions involving 25 different miRNAs and 21 inflammatory mRNAs (Table 5). Twenty-six of these predicted associations were at "conserved" sites (average context value of -0.35; average context percentage of 90) while the remaining 16 predicted associations were "poorly conserved" (average context value of -0.32; average

context percentage of 87). Comparison of these values with the 45 interactions of the confidence interval, 40 "conserved" and 5 "poorly conserved" (p <0.003257) (Table 6), suggests that the miRNAs targeting atherosclerotic inflammatory genes use statistically higher numbers of "poorly conserved" binding sites than general miRNAs.

In addition, we found that single tissue-targeted inflammatory gene miRNAs had lower frequencies of "conserved" interactions (12/24) than multiple tissue-targeted inflammatory gene miRNAs (14/18) (p = 0.063876) (not shown). This suggests that during inflammation, single tissue-targeted inflammatory gene miRNAs tend to use less "conserved" binding sites than multiple tissue-targeted inflammatory gene miRNAs. Moreover, we found that the 296 normally expressed miRNAs target the 33 inflammatory genes through 459 interactions, 327 "poorly conserved" and 132 "conserved", and contain binding features statistically identical to that of experimentally verified miRNAs. In comparison, the highly expressed miRNAs targeting inflammatory genes had 26 "poorly conserved" and 16 "conserved" binding interactions which was found to be statistically different from the experimentally verified miRNAs (p < 0.0001) (Table 6). These results suggest that normally expressed miRNAs use statistically higher numbers of "poorly conserved" binding sites than highly expressed miRNAs in targeting atherosclerotic inflammatory genes.

5. DISCUSSION

Previous research has established that numerous genes are up-regulated in atherogenesis through epigenetic or genetic transcriptional mechanisms (29). However, transcription-independent mechanisms have received far less scrutiny. In addition, recent research has demonstrated that changes in miRNAs expression patterns are connected to several pathological conditions including cardiovascular disease and atherosclerosis. These studies primarily focused on characterizing miRNAs which had been previously reported to have elevated expression in disease conditions in atherosclerosis disease models (30, 31). Thus, current miRNAs research has failed to address two important topics - how miRNAs regulate atherogenic inflammatory genes in a panoramic view and whether up-regulation of atherogenic inflammatory genes is the result of anti-inflammatory miRNAs down-regulation. In this study, we have developed a novel database mining approach in concert with a statistical analysis strategy established in our previous database mining publications (32-36). Our unique research has yielded several key findings. i) We discovered that the expression of 33 inflammatory genes (mRNAs) is up-regulated in atherosclerotic lesions and ii) that the mRNAs of those genes contain structural features in their 3'UTR for potential regulation by miRNAs. Furthermore, these structural features are statistically identical to experimentally verified 3'UTR miRNAs binding sites. It was also elucidated that iii) 21 out of the 33 inflammatory genes (64%) are targeted by highly expressed miRNAs while the remaining 12 inflammatory genes (36%) are

Table 3. Distribution of highly expressed miRNAs in atherosclerosis pertinent tissue and/or cell types

Cell/Tissue	miRNA
A. 25 miRNA had greater than normalized expression in 17 pertinent tissue and/or cell types	
B-Cell - CD19	miR-16, miR-29a, miR-29b, miR-29c, miR-150
Dendritic Cell	miR-15a, miR-16, miR-27a, miR-29b
Granulocytes	miR-15a, miR-16, miR-27a, miR-143
Heart	miR-1, miR-16, miR-27b, miR-30d, miR-143, miR-451
Hematopoietic stem cells - CD34	miR-15a, miR-16, miR-92a, miR-181a
Liver	miR-16, miR-22, miR-143
Monocytes	miR-15a, miR-16, miR-27a, miR-223
Natural Killer -CD56	miR-15a, miR-16, miR-29b, miR-150
Pancreatic	miR-7, miR-16, miR-26a, miR-29b, miR-141
Spleen	miR-16, miR-26a, miR-143
T-Cell CD4	miR-16, miR-29a, miR-29b
T-Cell CD4 Effector	miR-16, miR-29a, miR-29b, miR-32, miR-374a
T-Cell CD4 Naïve	miR-15a, miR-16, miR-29b, miR-30c, miR-150
T-Cell CD4 Memory	miR15a, miR-16, miR-30d, miR-30e, miR-140-5p, miR-150
T-Cell CD8	miR-16, miR-26a, miR-27a, miR-29b, miR-30e, miR-150
T-Cell CD8 Naïve	miR-16, miR-19b, miR-27a, miR-29b, miR-30e, miR-150
Thyroid	miR-16, miR-143

Ubiquitous Expression[1]	Global Expression[2]	Specific Expression[3]	Functional Expression[4]	Tissue
B. The 25 highly expressed miRNA and the pertinent tissue and/or cell types in which their expression is elevated				
		miR-1		Heart
		miR-7		Pancreas
	miR-15a			Dendritic cells, Granulocytes, HSC, Monocytes, NK, T-cell CD4 naïve, T-cell CD4 memory
miR-16				B-cell, Dendritic cell, Granulocytes, Heart, HSC, Liver, Monocyte, NK, Pancreas, Spleen, T-cell CD4, T-cell CD4 effector, T-cell CD4 memory, T-cell CD4 naïve, T-cell CD8, T-cell CD8 naïve, Thyroid
		miR-19b	miR-19b	T-cell CD8 naïve
		miR-22		Liver
	miR-26a			Pancreas, Spleen, T-cell CD8
	miR-27a			Dendritic cell, Granulocytes, Monocyte, T-cell CD8, T-cell CD8 naïve
		miR-27b		Heart
	miR-29a			B-cell, Pancreas, T-cell CD4, T-cell CD4 effector
	miR-29b			B-cell, Dendritic cell, NK, Pancreas, T-cell CD4, T-cell CD4 Effector, T-cell CD4 naïve, T-cell CD8, T-cell CD8 naïve
		miR-29c		B-cell
		miR-30c	miR-30c	T-cell CD4 naïve
	miR-30d			Heart, T-cell CD4 memory
	miR-30e			T-cell CD4 memory, T-cell CD8, T-cell CD8 naïve
		miR-32	miR-32	T-cell CD4 effector
		miR-92a		HSC
		miR-140-5p	miR-140-5p	T-cell CD4 memory
		miR-141		Pancreas
	miR-143			Granulocytes, Heart, Liver, Spleen, Thyroid
	miR-150			B-Cell, NK, T-cell CD4, T-cell CD4 memory, T-cell CD4 naïve, T-cell CD8, T-cell CD8 naïve
		miR-181a		HSC
		miR-223		Granulocytes, Monocytes
		miR-374a	miR-374a	T-cell effector
		miR-451		Heart

[1]Ubiquitously expressed in every tissue/cell type analyzed , [2]Expressed in multiple tissues/cell types analyzed, [3]Expressed in a single tissue/cell type analyzed, [4]Expressed in a certain functional/ activation status cell type

targeted by normally expressed miRNAs. iv) It was also established that 10 of the 21 highly expressed miRNA-targeted inflammatory genes (48%) were targeted by a single miRNA, suggesting the specificity of miRNA regulation. Meanwhile, 12 out of the 25 highly expressed miRNAs (48%) targeted single inflammatory genes while the other 13 miRNAs targeted multiple inflammatory genes. v) Finally, it was determined that the miRNAs targeting atherosclerotic inflammatory genes use statistically higher numbers of "poorly conserved" binding interactions than the control group of miRNAs used in the confidence interval. These results suggest that the miRNAs regulating atherosclerotic inflammatory genes have special features.

It is important to note that our database mining study differs from traditional human or mouse bioinformatic miRNAs binding prediction studies in that our approach analyzed experimentally verified miRNAs to establish data criteria (37). In addition, our list of

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Table 4. The indentified miRNAs have a range of pro-atherogenic molecular targets

MicroRNA	Predicted Target Gene[1]	Reported Molecular Target[2]	Reported Biological Activity	References (PMID)
miR-1	FYB	Delta-like-1	Cardiogenesis, Cardiac remodeling	18371447, 20003613, 20619221
miR-7	MKNK1	AKT Pathway, EGFR	Apoptosis, Cellular growth, Differentiation, Proliferation	18483236
miR-15a	IL10RA, MKNK1	BCL2	Apoptosis promotion	16166262
miR-16	C1QB, IL10RA, MKNK1	BCL2, VEGF	Apoptosis promotion, Angiogenesis	16166262, 17205120
miR-19b	ITGA6, TGFBR2	Unknown	Proliferation and survival of B and T cells	19887902, 20415654
miR-22	CSF1R	PTEN	Cell cycle regulation and survival	20523723
miR-26a	TAP1	PTEN	Cell cycle regulation and survival	19487573
miR-27a/b	CSF1, RGS1, RHOH	Unknown	Unknown	
miR-29a/b/c	TNFRSF1A	PI3K, CDC42, COL1A1, COL1A2, COL3A1, FBN1	Apoptosis promotion, Vascular fibrosis	19079265, 18723672
miR-30c/d/e	ITGA6, LYN	P53, Drp1, CTGF	Apoptosis promotion, Vascular fibrosis	20062521, 19096030
miR-32	ITGA6, PTPRC, PDE1A	Unknown	Unknown	
miR-92a	ITGA6, PDE1A	Unknown	Proliferation and survival of B and T cells	19887902, 20415654
miR-140-5p	C1R, FCGR2B	Unknown	Unknown	
miR-141	ITGA6, LCP2	Unknown	Unknown	
miR-143	ITGA6	ELK-1	Smooth muscles cell proliferation and differentiation	19578358, 20415654
miR-150	IRF8	C-Myb	Lymphopoiesis of B and/or T cells	17438277, 17923094
miR-181a	IRF8, VCAM	Unknown	B /T cell differentiation, T cell sensitivity and signaling strength	17549074, 17382377
miR-223	RGS1	Mef2c	Granulocyte differentiation	18278031
miR-374a	VCAM	Unknown	Unknown	
miR-451	CXCL16	Unknown	Erythroid differentiation	20513743

[1]Refer to Table 1 for full NCBI gene name [2]AKT - V-akt murine thymoma viral oncogene; EGFR - Epidermal growth factor receptor; BCL2 - B-cell CLL/lymphoma 2; VEGF - Vascular endothelial growth factor; PTEN - Phosphatase and tensin homolog; PI3K - Phosphoinositide 3-kinase; CDC42 - Cell division cycle 42; COL1A1 - Collagen, type I, alpha 1; COL1A2 - Collagen, type I, alpha 2; COL3A1 - collagen, type III, alpha 1; FBN1 - Fibrillin 1; P53 - Tumor protein p53 ; Drp1 – dynamin-related protein; CTGF - Connective tissue growth factor ; ELK-1 - ELK1, member of ETS oncogene family; C-Myb - V-myb myeloblastosis viral oncogene homolog; Mef2C - Myocyte enhancer factor 2C

inflammatory genes was not collected from irrelevant inflammatory pathologies, (38) rather it was derived from previous publications of microarray experiments on atherosclerotic samples (18) ensuring pathophysiological relevance. It should also be mentioned that our studies excluded the possibility that overexpressed pro-inflammatory miRNAs may result in down-regulation of certain atherosuppresive genes. This exclusion was made with the intention of addressing the issue separately in the near future.

Based on their cell/tissue expression and target genes, the anti-atherogenic miRNAs identified in this study can fulfill their anti-inflammatory function through several mechanisms such as inflammatory cell regulation, apoptosis control, smooth muscle cell regulation, extracellular matrix remodeling, as well as cell proliferation and differentiation (Table 4B). Inflammatory cell regulation, especially the aptitude to modulate lymphocyte proliferation and sensitivity, would prove critical in curbing the development of atherosclerosis. Several of the miRNAs that we elucidated have physiological activity in this area including miR-19b, miR-92a, miR-150, miR-181a, and miR-223. For

example, with elevated expression in the lymph nodes, spleen, and thymus, miR-150 has been implicated in the dictation of lymphopoiesis of B and/or T cells (39). This governing is mechanistically possible through interactions with the transcription factor c-Myb, which plays a key role in lymphopoiesis (40). Meanwhile, hematopoietic-specific miR-181a has been demonstrated to regulate the differentiation of B and T cells (41) and influence T cell sensitivity and signaling strength (42). It is easy to comprehend how this modulation could have far-reaching impact on the immune system and autoimmune development. Since apoptosis is a contributing factor to the development of atherosclerotic plaques and general inflammation, it is another potential area for anti-inflammatory miRNAs control (43). It has been reported that endothelial apoptosis may contribute to the initiation of atherosclerosis while vascular smooth muscle and macrophage apoptosis leads to plaque instability (44). Our experimentally-generated list yielded several miRNAs - miR-15a, miR-16, miR-29a, miR-29b, miR-29c, miR-30c, miR-30d, and miR-30e, involved in apoptosis dictation. This control is best illustrated by subfamily members of miRNA-29 and miRNA-30

Structural evidence of anti-atherogenic microRNAs

Table 5. The gene/miRNA interactions that were statistically identical to experimentally verified miRNA based on their context values and %

Gene	miRNA	Context Value	Context %	Conservation
C1QB	miR-16	-0.22	79	Poorly Conserved
C1R	miR-140-5p	-0.47	99	Poorly Conserved
CSF1	miR-27b	-0.26	83	Conserved
CSF1	miR-27a	-0.26	83	Conserved
CSF1R	miR-22	-0.37	97	Conserved
CXCL16	miR-451	-0.47	99	Poorly Conserved
FCGR2B	miR-140-5p	-0.25	82	Poorly Conserved
FYB	miR-1	-0.49	99	Conserved
IL10RA	miR-15a	-0.25	85	Conserved
IL10RA	miR-16	-0.25	84	Conserved
IRF8	miR-150	-0.29	94	Poorly Conserved
IRF8	miR-181a	-0.28	80	Poorly Conserved
ITGA6	miR-19b	-0.36	88	Conserved
ITGA6	miR-32	-0.39	93	Conserved
ITGA6	miR-92a	-0.38	92	Conserved
ITGA6	miR-30e	-0.46	96	Conserved
ITGA6	miR-30d	-0.46	96	Conserved
ITGA6	miR-30c	-0.47	96	Conserved
ITGA6	miR-143	-0.36	94	Conserved
ITGA6	miR-141	-0.33	90	Poorly Conserved
LCP2	miR-141	-0.36	92	Poorly Conserved
LYN	miR-30d	-0.39	89	Conserved
LYN	miR-30c	-0.39	89	Conserved
LYN	miR-30e	-0.39	89	Conserved
MKNK1	miR-7	-0.24	86	Conserved
MKNK1	miR-15a	-0.39	96	Conserved
MKNK1	miR-16	-0.4	96	Conserved
PDE1A	miR-92a	-0.39	93	Poorly Conserved
PDE1A	miR-32	-0.39	92	Poorly Conserved
PTPRC	miR-32	-0.23	76	Poorly Conserved
RGS1	miR-27a	-0.23	79	Conserved
RGS1	miR-27b	-0.23	78	Conserved
RGS1	miR-223	-0.36	92	Conserved
RHOH	miR-27b	-0.23	78	Poorly Conserved
RHOH	miR-27a	-0.23	78	Poorly Conserved
TAP1	miR-26a	-0.38	93	Poorly Conserved
TGFBR2	miR-19b	-0.3	81	Conserved
TNFRSF1A	miR-29c	-0.32	92	Conserved
TNFRSF1A	miR-29a	-0.32	92	Conserved
TNFRSF1A	miR-29b	-0.32	91	Conserved
VCAM1	miR-181a	-0.31	85	Poorly Conserved
VCAM1	miR-374a	-0.35	86	Poorly Conserved

Table 6. Comparison of miRNA lists using the Pearson's chi square test

Group	miRNA-mRNA Interactions	Conserved	Poorly Conserved	
524 miRNA Before CI[1] Filtration	1368	198	1170	<0.0001
296 miRNA After CI[1] Filtration	459	132	327	
296 miRNA After CI[1] Filtration	459	132	327	<0.0001
25 Highly Expressed miRNA	42	26	16	
25 Highly Expressed miRNA	42	26	16	0.0033
45 miRNA with the CI[1]	45	40	5	

[1]CI - Confidence Interval

which are able to target p53, a protein known to induce apoptosis. Smooth muscle cells (SMCs) have demonstrated the capability to express lipid uptake receptors, secrete extracellular matrix proteins, and present adhesion molecules for the adhesion of monocytes and lymphocytes thereby contributing to atherosclerosis development and progression (45). Yielded by our search results, miRNA-143 is known to inhibit SMC proliferation and effect differentiation (16) through interactions with transcription factors which are essential for SMC development and growth

(12). Another mechanistic target is extracellular matrix modeling and fibrotic proliferation which are essential elements in the development of atherosclerotic lesions and plaque stability (46). Both miR-29 and miR-30 family members have been implicated in modulating aspects of fibrosis. Involved in the repression of several fibrillins, collagens, and elastins, miR-29 has been reported to play a key role in cardiac fibrosis (47). Alternatively, miR-30 has been shown to directly repress the expression of the profibrotic protein

Table 7. The down-regulation of miRNAs upon pro-atherogenic factor stimulation as supported by published literature

MicroRNA	LPS Stimulation	Ox-LDL	Cytokines
miR-1			
miR-7			
miR-15a		19377067[1]	
miR-16			
miR-19b			
miR-22			
miR-26a			19823581[1]
miR-27a/b			
miR-29a/b/c	20890893[1]		20890893[1]
miR-30c/d/e			
miR-32		19377067[1]	
miR-92a			
miR-140-5p			19541842[1]
miR-141			
miR-143		19377067[1]	
miR-150			19823581[1]
miR-181a			
miR-223			
miR-374a			
miR-451			

[1]Pubmed IDs

connective tissue growth factor (48). The regulation of cell proliferation and differentiation also requires finely tuned gene expression control for normal physiological functioning. Our study found several miRNAs – miR-1, miR-7, miR-22, miR-26a, and miR-451 involved in this role. Exemplifying this control and critical for cardiogenesis, miR-1 is a vital participant of muscle proliferation and differentiation. Additionally, miR-1 expression has been found to be reduced following cardiac hypertrophy indicating participation in cardiac remodeling (49). Meanwhile, miR-7 has been shown to inhibit the epidermal growth factor (EGF) receptor. This receptor and its signaling are responsible for controlling cellular growth, differentiation, and proliferation through interactions with the EGF receptor.

Previous research has shown that miRNAs participate in modulating atherosclerosis-related processes including hyperlipidemia (miRNA-33, miRNA-125a-5p), hypertension (miRNA-155), plaque rupture (miRNA-222, miRNA-210), and atherosclerosis itself (miRNA-21, miRNA-126) (30). However, the question of whether certain miRNAs can play a role in preventing disease development remains unknown. One of the most interesting findings from our study is that the 25 miRNAs that are highly expressed under normal untreated conditions target 21 out of the 33 atherosclerosis-up-regulated inflammatory genes (64%). This important result suggests a novel mechanism where a group of highly expressed anti-inflammatory miRNAs suppress the up-regulation of proatherogenic inflammatory genes under normal physiological conditions. It has been well established that miRNAs play important roles in fine-tuning developmental processes and participate in the development of diseases such as inflammation and cancer. Our results are the first to suggest that miRNAs may play a protective role by suppressing proatherogenic genes and maintaining

healthy artery functional status (see our working model in Figure 2). Our conclusion is supported by other publications (Table 7) which show that 7 out of the 20 miRNAs (35%) identified in this study were down-regulated by various proatherogenic factors. For example, the proatherogenic risk factor lipopolysaccharide (LPS) induces the down-regulation of miRNA-29a/b/c, while the proatherogenic factor oxidized low density lipoprotein (oxLDL) down-regulates miRNA-15a, miRNA-32, and miRNA-143. All four miRNAs were identified in our study. More interestingly, miRNA-143 appears to be down-regulated in neointimal formation models (50) and represses the atherogenic proliferative response of vascular smooth muscle cells to injury (51). Furthermore, proatherogenic inflammatory cytokines have been shown to induce down-regulation of miRNA-26a, miRNA-29a/b/c, miRNA-140-5p, and miRNA-150. In addition to miRNAs identified in this study, Chen et al. (2009) showed that inhibition of endogenous miRNA-125a-5p levels in THP-1 cells significantly increases the secretion of inflammatory cytokines including transforming growth factor (TGF)-beta, tumor necrosis factor (TNF)-alpha, interleukin (IL)-2, and IL-6. Suppression of miRNA-125a-5p also led to increases in the expression of macrophage scavenger receptors (LOX-1 and CD68) which resulted in increased lipid uptake (52).

In conclusion, our results indicate for the first time that the up-regulation of certain inflammatory genes in atherosclerotic plaques may be the result of inflammatory-suppressing miRNAs down-regulation. This study provides insight into a novel miRNAs protection mechanism involved in suppressing pro-atherogenic inflammatory genes lending individual protection robustness against the perturbation of proatherogenic risk factors. This study has also pointed out potential new approaches in microRNAs-based therapeutics.
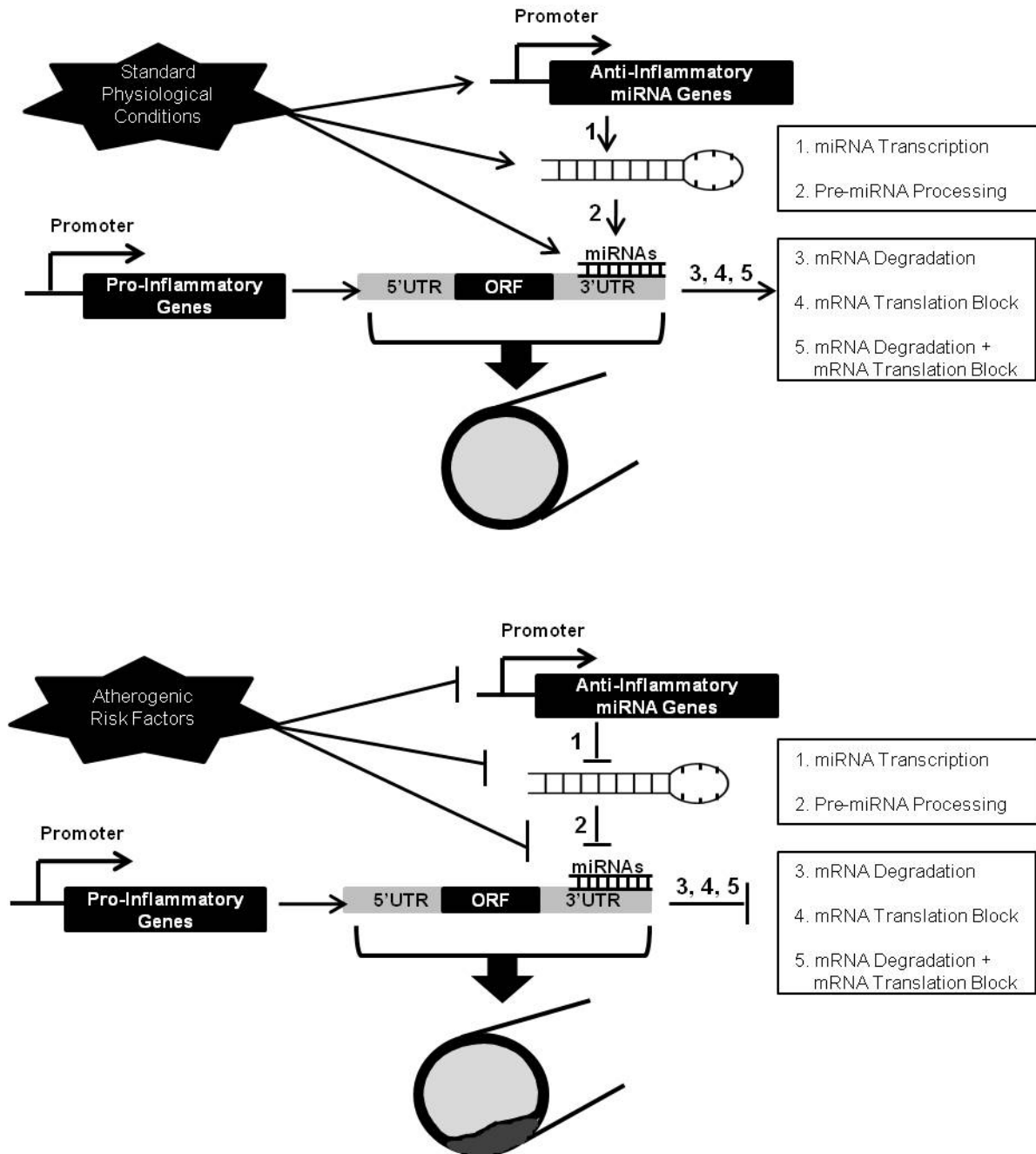
Figure 2. A. Working model of pro-inflammatory gene suppression by miRNA resulting in no atherosclerosis lesion formation. B. Working model of pro-atherogenic risk factor suppression of miRNA expression resulting in inflammatory gene expression and atherosclerosis lesion formation.

7. DISCLOSURES

The authors have no conflicts of interest.

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