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Interleukin-27 treated human macrophages induce the expression of novel microRNAs which may mediate anti-viral properties

Sanjay Swaminathan[1], **Xiaojun Hu**[1], **Xin Zheng**[1], **Yuliya Kriga**[2], **Jyoti Shetty**[2], **Yongmei Zhao**[3], **Robert Stephens**[3], **Bao Tran**[2], **Michael W. Baseler**[1], **Jun Yang**[1], **Richard A. Lempicki**[1], **Dawei Huang**[1], **H. Clifford Lane**[4], and **Tomozumi Imamichi**[1]

[1]Applied and Developmental Research Directorate, Science Application International Corporation (SAIC)-Frederick, Inc., Frederick National Laboratory for Cancer Research (FNLCR), Frederick, MD 21702, USA

[2]Advanced Technology Program Directorate, SAIC-Frederick, Inc., FNLCR, Frederick, MD, 21702

[3]Information Systems Program Directorate, SAIC-Frederick, Inc., FNLCR, Frederick, MD, 21702

[4]Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

Abstract

Interleukin-27 (IL-27) is a pleiotropic cytokine which plays important and diverse roles in the immune system. We have previously demonstrated that IL-27 induces potent anti-viral effects against HIV-1, HIV-2, SIV, HSV-2, KSHV and influenza viruses in macrophages. This induction occurred in an Interferon (IFN) independent manner and involved down regulation of SPTBN1. MicroRNAs (miRNAs) are critical regulators of mRNA translation and turnover. There have been reports that some miRNAs inhibit viral replication. In this study, we hypothesized that IL-27 could induce the expression of novel miRNAs in macrophages which may have functional relevance in terms of anti-viral activity and primary monocytes were differentiated into macrophages using either M-CSF (M-Mac) or a combination of M-CSF and IL-27 (I-Mac) for seven days. Following this, total RNA was extracted from these cells and deep sequencing was performed, in parallel with gene expression microarrays. Using the novel miRNA discovery software, miRDeep, seven novel miRNAs were discovered in these macrophages. Four of which were preferentially expressed in I-Mac (miR-SX1, -SX2, -SX3 and -SX6) whilst three were detected in both M-Mac and I-Mac (miR-SX4, -SX5 and -SX7). The expression of six of the seven novel miRNAs was highly correlated with qRT-PCR using specific primer/probes designed for the novel miRNAs. Gene expression microarray further demonstrated that a number of genes were potentially targeted by these differentially expressed novel miRNAs. Finally, several of these novel miRNAs (miR-SX1, -SX4, -SX5, -SX6 and -SX7) were shown to target the open reading frames of a number of

Corresponding author: Dr Tomozumi Imamichi Laboratory of Human Retrovirology Building 550, Room 126, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research 1050 Boyles St. Frederick, MD, USA, 21702 Phone: +1-301-846-5450 timamichi@mail.nih.gov.

Conflicts of Interest
The authors report no conflicts of interest.

viruses (including HSV-1, HSV-2 and HHV-8) which may partially explain the anti-viral properties observed.

Keywords

Interleukin-27; macrophages; MicroRNAs; HIV; HSV-1; HSV-2; HHV-8

Introduction

Interleukin 27 (IL-27) is a member of the IL-12 family of cytokines, and plays a multifaceted role in the immune system [1]. The conventional view is that IL-27 acts predominantly as an anti-inflammatory cytokine [2]. More recently, a number of studies have shown that IL-27 also possesses significant anti-viral properties, particularly against HIV-1. The mechanism of the IL-27 mediated anti-HIV activity is thought to be interferon (IFN) independent and has been demonstrated in a number of cell types including CD4+ T cells [3], macrophages [3,4,5,6] and dendritic cells [7]. In the studies investigating the role of IL-27 in macrophages, monocytes were differentiated into macrophages either with M-CSF alone (M-Mac) or in the presence of IL-27 and M-CSF (I-Mac). The macrophages treated with IL-27 (I-Mac) displayed significant HIV-1 resistance compared to M-Mac along with resistance to HIV-2 HSV-2, HHV-8 and influenza virus infections [6]. In our previous work, we have demonstrated that SPTBN1, a member of spectrin family proteins, plays a key role as an essential host factor in macrophages for HIV-1 infection [6]. In I-Mac, IL-27 treatment down-regulated the expression of SPTBN1 and subsequently suppressed interaction with HIV core protein, followed by inhibition of the initiation of HIV-1 reverse transcription at an early stage of infection. In contrast, the mechanism of anti-viral effects against other viruses in I-Mac is not well understood.

The discovery that small 19-22 nucleotide long non-protein coding RNAs, termed microRNAs (miRNAs), are critical regulators of mRNA translation and turnover, has revolutionized our understanding of biology. These miRNAs bind to areas in the 3’ untranslated region (3’ UTR) of messenger RNA (mRNA) via the RNA induced silencing complex (RISC) which ultimately leads to mRNA degradation or translational repression [8]. More than half of all mRNAs are thought to contain 3’UTRs that are potentially regulated by miRNAs [9]. The latest Sanger miRNA database v19 (mirbase.org) contains 2042 human miRNAs, with the numbers steadily increasing. Cytokines, particularly Type I interferons (IFN), have been shown to modulate the expression of endogenous miRNAs. These IFN-up-regulated miRNAs possess the ability to bind to the open reading frame (ORF) of viral RNA such as that found in Hepatitis C and may exert some direct anti-viral properties [10]. To further investigate the anti-viral properties of IL-27 we tested the hypothesis that IL-27 treatment of macrophages might induce the expression of novel miRNAs that may affect the antiviral function in I-Mac.

Materials and Methods

Ethics Statement

Ethics approval for obtaining leukopacks was granted by the institutional review board of the NIH.

Generation of Macrophages

CD14+ monocytes were isolated from healthy donor peripheral blood enriched leukopacks (Blood Bank, National Institute of Health, Bethesda, MD, USA) using MACS CD14

MicroBeads (Miltenyi Biotec, Auburn, CA, USA). Monocytes were differentiated into macrophages using M-CSF (R&D Systems, Minneapolis, MN, USA) (M-Mac) or a combination of M-CSF and IL-27 (R&D Systems) (I-Mac) as described in [6].

Preparation of RNA and QC for RNA

Total RNA was extracted using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany). Prior to RNA sequencing, samples were subjected to analysis by the Agilent Bioanalyzer RNA Nano chip and the small RNA chip (Agilent Technologies, Santa Clara, CA, USA) to confirm RNA purity and quality (all four samples had a RNA integrity number of 10).

Illumina RNA-seq

Four microRNA libraries were prepared using the Illumina TruSeq Small RNA Sample Preparation protocol according to the manufacturer's instructions. Briefly, the specifically modified RNA 3’ adaptor, which has a high affinity for Dicer cleavage products, and 5’ adaptor were aligned to the gel purified small RNAs, followed by reverse transcription and library enrichment by PCR amplification. The size, purity, and concentration of the final cDNA library were validated using an Agilent Bioanalyzer 2100. Samples were sequenced on Illumina GAIIx platform with 36 cycles using TruSeq SBS Kit v5-GA chemistry.

Read processing and mapping

The raw reads were trimmed against Illumina adapters, and rRNA, primers, mitochondria contaminations, and filtered by minimum length 15, maximum length 32 and phred-score 20 using fastq-mcf (code.google.com/p/ea-utils/wiki/FastqMcf). The reads were collapsed to tags by our own perl script. Tags containing 1 read were treated as machine error and removed. The clean tags were mapped to the hg19 build of the human genome (UCSC Genome Browser) and human mature miRNA (miRbase v19) using novoalign (http://www.novocraft.com/) with 2 mismatches. The genetic locations of tags were annotated using ANNOVAR [11].

Identification of novel miRNAs

Qualified tags were inputted into the publicly available miRNA discovery program, miRDeep [12] to identify novel miRNAs. For each sample, around 600 novel miRNAs were identified. All miRNAs were summarized together according to their genetic locations and mature sequences. If the novel miRNA was only identified in one sample, the novel miRNA was excluded in the summary. Finally, 500 novel miRNA candidates were collected. To determine their abundance, each candidate was mapped to novel mature sequences using novoalign with miRNA mode and 2 mismatches. Reads counts were calculated using our own scripts from novoalign mapping result, and then normalized by DESeq [13]. The four top differentially expressed miRNAs and 3 top high abundance miRNAs were further prioritized based on (i) the occurrence of reads on the stem of a predicted stem-loop structure with minimum free energy less than -25 kcal/mol; (ii) a mature sequence highly conserved between species (checked in UCSC genome browser); (iii) a mature sequence falling within one arm and lacking large internal loops or bulges; and (iv) a mature sequence is not available in Rfam (rfam.sanger.ac.uk).

Prediction of miRNA targets and viruses targeted by miRNAs

To predict the mRNA targets of the novel miRNAs, the 3’UTRs of 571 genes selected from the microarray experiment and extracted from UCSC genome were analyzed by two programs were used: TargetScan [14] with context+score <-0.10 cutoff, and miRanda [8] with energy <-20 Kcal/mol cutoff. For stringency purposes, the targets had to be predicted by both programs to be accepted as a genuine target of the novel miRNA.

To predict which novel miRNAs might be targeting viral sequences, 4537 viral reference sequences downloaded from NCBI (ftp://ftp.ncbi.nih.gov/refseq/release/viral/) were scanned by miRanda with minimum free energy <-20 kcal/mol. Binding sites with a score ≥100 were selected as potential novel miRNA targets. Viruses infecting human cells/organism were targeted in the final analysis.

Microarrays

The gene expression profiles in M-Mac and I-Mac were obtained using the human GeneArray ST 1.0 microarray (Affymetrix, Santa Clara, CA, USA) as described previously [6,7]. Both microarray and sequencing data has been uploaded to the Gene Encyclopedia Omnibus (GEO): http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44957

Real-time PCR

To confirm expression of novel miR-SX2, -SX3, -SX4, -SX5, -SX6 and -SX7, qRT-PCR was performed. The novel miRNA probes were custom made using the Custom Small RNA assay platform provided by Applied Biosystems (Carlsbad, CA, USA). A probe for miR-SX1 was unable to pass the quality control from production in the company due to a very high GC content (84.2 %) of miR-SX1 (Table 1), and therefore a custom-made probe could not be made for this particular miRNA. Reverse transcription (RT) of RNA, followed by real-time PCR was performed using commercial kits from Applied Biosystems. For miR-SX5, 10 ng of total RNA was used for the specific RT reaction. Due to an inability to detect product after PCR using this starting amount of RNA, we increased the total starting RNA total to 100 ng for the initial RT reaction for miR-SX2, -SX3, -SX4, -SX6 and -SX7. The RT and PCR cycling protocols were followed by recommended by the manufacturer as described in [15].

Statistical Analysis

Statistical differences in novel miRNA expression between M-Mac and I-Mac were calculated by the 2 tailed student t test using Prism 5 for Windows (GraphPad Software, Inc.). P values <0.05 were considered statistically significant.

Results

RNA sequencing of macrophages reveals novel miRNAs

Monocytes from two healthy donors were isolated from PBMCs and differentiated using M-CSF alone (M-Mac) or in the presence of both M-CSF and IL-27 (I-Mac). After seven days, the monocytes were fully differentiated into macrophages and total RNA was extracted.

All samples had good yields with over 23 million pass filter reads generated and excellent base call qualities with over 95% of the bases having Q30 or above. After cleaning, around 30% of reads were kept except M-Mac Donor 1 which only had 10.9%. To evaluate the data quality, the clean reads were mapped to the human genome (hg19 from UCSC genome), 96.3 – 98.7% of the reads showed alignment to the human genome. Around 98% of the reads were located in intronic or intergenic regions. The clean reads were also “blasted” against all known human mature microRNAs downloaded from miRBase v19. Between 83.1-86.3% of the reads were aligned to the human mature miRNAs with alignment length ≥18 bases plus 2 mismatches. We found a total of 701 known miRNAs in the four samples and seven novel miRNAs. The average number of sequence reads for known miRNAs in M-Mac was 7,426.5 (range: 0 – 1,067,644), whilst in I-Mac the average number of reads were 8,344.5 (range: 0 – 5,951,676). For novel miRNAs, the average number of sequence reads for M-Mac was 5,601 reads (range: 5 – 72,328), whilst for I-Mac the average number of reads was 8,147.5 (range: 43 – 55,571) (Figure 1A and 1B and supplementary Table 1).

Four novel miRNAs are over-expressed in I-Mac

As mentioned above, a total of seven novel miRNAs were discovered using the miRDeep program [12]: miR-SX1, -SX2, -SX3, -SX4, -SX5, -SX6 and -SX7 (Table 1). The novel miRNAs were traced back to their possible genomic locations to see whether secondary stem-loop structures could be formed. As shown in Figure 2, these seven miRNAs could all form appropriate stem loop structures as shown by the predicted secondary structures. In terms of abundance (as judged by the number of reads), miR-SX5 was the most highly expressed novel miRNA, followed (in descending order) by miR-SX1>-SX7>-SX4>-SX2>-SX6>-SX3. Four novel miRNAs (miR-SX1, -SX2, -SX3, -SX6) were noted to be much higher in I-Mac compared to M-Mac (Figure 1C – 1I). Only one of these miRNAs failed to show any level of expression in M-Mac (miR-SX3). Using the UCSC genome browser, the novel miRNAs were found to be broadly conserved in a variety of vertebrates, particularly mammals (Supplementary Figures 1-4).

qRT-PCR validation of novel miRNAs

To validate the expression levels of the identified novel miRNAs, custom made Taqman probes were made for six of the seven miRNAs, for subsequent analysis with PCR. Due to the very high GC content of miR-SX1, custom made probes could not be made for this particular miRNA. The PCR validation showed a close correlation with the sequencing results (Figure 3). Expression levels for miRNAs-SX3 and SX6 were much higher in I-Mac than M-Mac. Like the sequencing results, no miR-SX3 was found in M-Mac. The most abundant novel miRNA in I-Mac, as was the case with the sequencing results, was miR-SX5, followed by miR-SX7>-SX4>-SX2>-SX3>-SX6. These results were similar to the sequencing results, apart from the juxtaposition of miRNAs-SX3 and SX6, which were noted to be lowly expressed using both platforms. Monocytes from a total of five donors were used to make M-Mac and I-Mac and PCR was performed to verify the levels of these novel miRNAs in the extra donors. In these five donors, miRNA-SX3 ($p<0.01$) and miR-SX6 ($p=0.01$) were significantly higher in I-Mac compared to M-Mac.

Predicted mRNA targets of miRNAs up-regulated in I-Mac

An Affymetrix gene expression microarray was performed in parallel with the RNA sequencing with three donors used (M-Mac versus I-Mac for each donor). Table 2 lists mRNA targets for the novel miRNAs that were up-regulated in I-Mac (miR-SX1, -SX2, -SX3 and -SX6). The observation that those genes were correspondingly reduced in the microarray suggests that these miRNAs may be playing a role in controlling the expression of these genes. A total of 26 genes were targeted by the novel miRNAs. Two mRNA targets (AFAP1L1 and UCHL1) were targeted by two separate miRNAs (miR-SX2 and SX6) and both were down-regulated more than three-fold in I-Mac compared to M-Mac. Further functional studies with novel miRNA mimics will be required to verify if they indeed can modulate these genes in macrophages.

Viruses targeted by novel miRNAs

One of the main purposes of this study was to investigate if any of the discovered novel miRNAs may have direct anti-viral properties. Previous work has shown that I-Mac has broad anti-viral activity in terms of inhibiting HIV-1, HIV-2, Influenza, HSV-2 and HHV-8 [6]. In this study, of the seven novel miRNAs, five were found to target pathogenic viruses in humans (miR-SX1, -SX4, -SX5, -SX6 and -SX7). Two failed to find any viral matches (miR-SX2 and -SX3). As shown in Table 3, the novel miRNAs targeted a number of viruses including a number from the human herpes group of viruses (HSV-1, HSV-2, HHV-4 and HHV-8). Of note, there were no matches found for several viruses for which I-Mac has been shown to restrict replication, including HIV-1, HIV-2, influenza and SIV [6].

Discussion

In our previous work, we have demonstrated that IL-27 treatment induces macrophages to become resistant to infection by several viruses including HIV-1, HIV-2, SIV, HSV-2, KSHV (HHV-8) and the influenza virus. However, the molecular mechanism of the anti-viral effect is under investigation. We hypothesized that IL-27 may induce some novel miRNAs which potentially targeted these viruses to explain their resistance. In this study, we discovered seven novel miRNAs in macrophages using a deep sequencing platform. Four of these novel miRNAs (miR-SX1, -SX2, -SX3 and -SX6) were highly expressed in IL-27 treated macrophages (I-Mac), whilst three novel miRNAs were found in both M-Mac and I-Mac (miR-SX4, -SX5 and -SX7). These seven novel miRNAs are broadly conserved within vertebrates, particularly mammals. The induction of these four miRNAs in I-Mac was associated with a concurrent down-regulation of 26 putative mRNA targets, as detected by an Affymetrix microarray. Of interest, miR-SX1, -SX4, -SX5, -SX6 and -SX7 were able to potentially target the ORF of a number of viruses (such as HSV-1, HSV-2 and HHV-8), suggesting that part of the anti-viral phenotype in I-Mac might be related to the presence of these novel miRNAs, although more functional studies will need to be performed to extend the current work.

Prior work has demonstrated that IL-27 is a highly potent anti-viral agent, particularly in HIV-1 infection in CD4+ T cells, macrophages and dendritic cells [3,4,5,6,7]. IL-27 has also been shown to possess significant anti-viral properties against a number of other viruses including HIV-2, SIV, influenza, HSV-2 and HHV-8 [6]. One mechanism appears to be the down-regulation of SPTBN1 [6], but it is likely that other mechanisms may also play a role. Previous work has demonstrated that miR-32 can serendipitously target the ORF of the retrovirus, primate foamy virus [16] in human cells, demonstrating that it is plausible that miRNAs may be able to target viral sequences in mammalian cells. Further, using Dicer-deficient mice with reduced overall levels of miRNAs, vesicular stomatitis virus (VSV) infection was seen to be enhanced and this was shown to be secondary to decreased levels of miR-24 and miR-93 which targeted key viral genes. The down-regulation of these miRNAs led to increased viral replication [17]. Endogenous miRNAs may also be able to target critical genes of the HIV-1 genome [18] and keep HIV-1 in a latent stage in resting CD4+ T cells [19] (reviewed in [20]). In the current study, a number of the novel miRNAs identified in macrophages may potentially bind to complementary sequences of the ORF of a number of viruses. Two of the novel miRNAs induced in I-Mac (miR-SX1 and -SX6) had potential binding sites within the ORF of HSV-2 and HHV-8. This may explain to some degree the decreased replication of these viruses seen in I-Mac compared to M-Mac. Further work in infected macrophages over-expressing these novel miRNAs will need to be performed before this can be definitively concluded.

A number of viruses which were inhibited in I-Mac (including HIV-1, HIV-2, SIV and influenza) were not directly targeted by the novel miRNAs induced by IL-27, suggesting other mechanisms may be at play. We recently demonstrated that a spectrin family protein, SPTBN1 is an essential host factor in HIV-1 infection in macrophages and IL-27 down-regulates this protein in I-Mac [6]. In this study, we demonstrated that I-Mac induced novel miRNAs (-SX1, -SX2, -SX3 and -SX6) that down-regulate additional host genes that may play a role in viral pathogenesis. In particular, two separate novel miRNAs (-SX2 and -SX6) targeted the same gene, AFAP1L1. AFAP1L1 is a member of the actin-filament associated protein (AFAP) family of adaptor proteins and has been shown to bind to actin [21]. A number of studies have indicated that the actin cytoskeleton may play an important role in the pathogenesis of HIV-1 and other viruses [22,23,24] (the role of actin and viral infections is reviewed in reference [25]). Therefore it is plausible that the significant down-regulation

of an actin binding protein, such as AFAP1L1 in I-Mac, may also play a role in the mediating anti-viral effect by IL-27.

In summary, we discovered seven novel miRNAs (miR-SX1, -SX2, -SX3, -SX4, -SX5, -SX6 and -SX7) in human macrophages. Three miRNAs (miR-SX4, -SX5 and -SX7) were expressed in both M-Mac and I-Mac, whilst miR-SX1, -SX2, -SX3 and -SX6 were preferentially induced in IL-27 treated macrophages (I-Mac). Functional studies aimed at characterizing the effects of these novel miRNAs on various viral infections and cell type specificity in the expression of the miRNAs will help to further delineate the role of miRNAs in host defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Using deep sequencing, we identified seven novel miRNAs in macrophages
- Four of the seven novel miRNAs were up-regulated with IL-27 treatment
- Up-regulation of the novel miRNAs with IL-27 was shown to decrease mRNA targets
- In-silico programs suggested some of the novel miRNAs could target human viruses

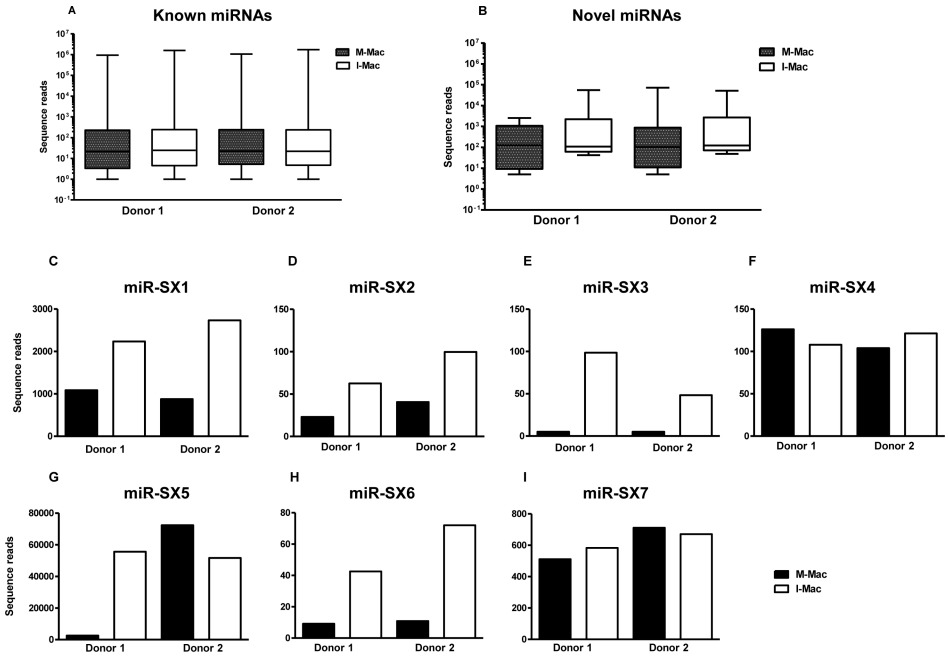

Figure 1. Number of reads of novel miRNAs in the RNA sequencing data

(A,B) A boxplot summarizes the sequence reads for both known (A) and novel miRNAs (B). The number of reads by RNA sequencing (on the Y axis) of each novel miRNA was plotted against the sample type (C-I). Four novel miRNAs (miR-SX1, -SX2, -SX3 and -SX6) were shown to have higher levels in I-Mac compared to M-Mac whilst the remaining miRNAs (-SX4, -SX5 and -SX7) were shown to have similar levels between M-Mac and I-Mac.

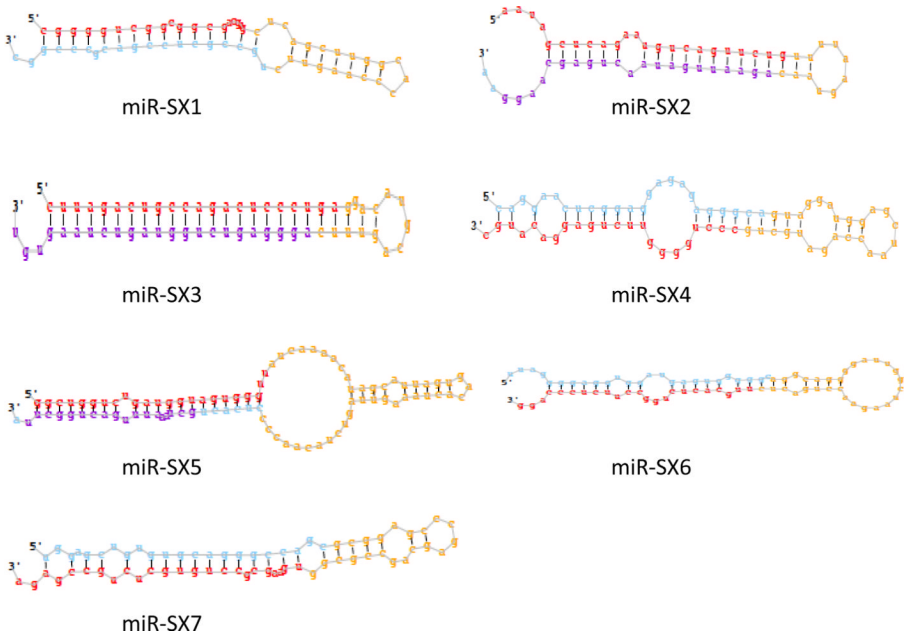

Figure 2. Secondary structures of the novel miRNAs

All novel miRNAs were able to form stem-loop hairpin structures but each novel miRNA varied in the location, number and size of bulges. Red indicates the mature sequence; blue indicates the star sequence; yellow indicates loop structure, purple indicates star sequence.

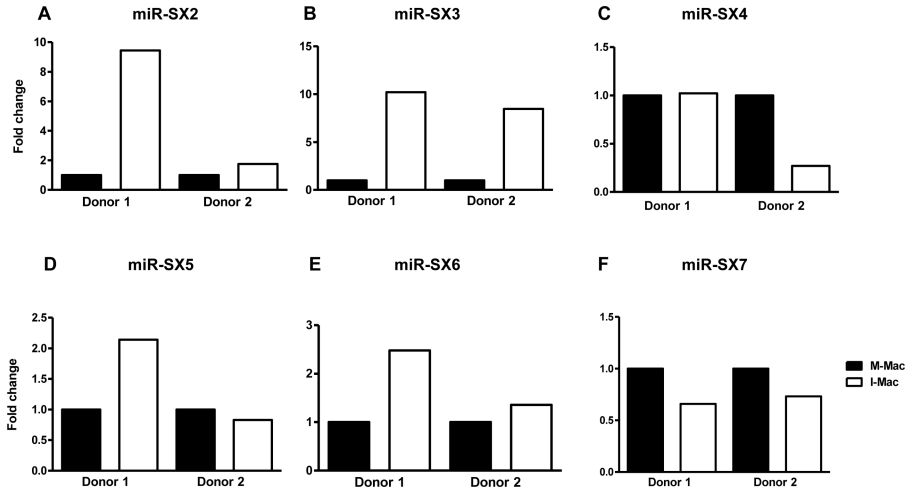

Figure 3. qRT-PCR validation of novel miRNA

(A-F) Using custom made primers for six of the seven novel miRNAs, validation of the expression levels of these miRNAs were carried out using qRT-PCR confirming that miRNAs-SX2, SX3 and SX6 were more highly expressed in I-Mac compared to M-Mac.

Table 1

Sequence and genomic location of novel miRNAs[a]

Novel miRNA	Official miRBase name	GenBank Accession number	Mature sequence 5′-3′	Precursor genomic location	Precursor minimum free energy(kcal/mol)
SX1	hsa-miR-7704	KC832801	CGGGGUCGGCGGCGACGUG	Chr2:177053571-177053630	-30.8
SX2	hsa-miR-7705	KC832802	AAUAGCUCAGAAUGUCAGUUCUG	Chr8:101715195-101715252	-25.2
SX3	hsa-miR-7702	KC832799	CUUAGACUGCCAGACUCCCUGA	Chr9:114033435-114033494	-43.7
SX4	hsa-miR-6852	KC832803	CCCUGGGGUUCUGAGGACAUGC	Chr9:35710711-35710782	-29.9
SX5	*	KC832805	GGCUGGUCUGAUGGUAGUGGGUU	Chr13:60761874-60761964	-27.9
SX6	hsa-miR-7703	KC832800	UUGCACUCUGGCCUUCUCCCAGG	Chr14:24612697-24612774	-30.2
SX7	hsa-miR-7706	KC832804	UGAAGCGCCUGUGCUCUGCCGAGA	Chr15:85923827-85923894	-28.7

[a]The novel miRNAs were identified using miRDeep[13], DESeq[14] and Rfam (rfam.sanger.ac.uk) as described in the materials and methods.

*No official miRBase name was assigned yet to this novel small RNA as it may be fragment from a Y RNA

Table 2

Putative targets for novel miRNAs[a]

Novel miRNA	Target gene name	mRNA FC ↓ I-Mac (Log2)[b]	miRNA FC↑ I-Mac (Log2)[b]
SX1	GFOD1	-0.62	1.34
SX1	MARCH3	-0.64	1.34
SX1	SHC3	-0.58	1.34
SX2	ADRA2B	-0.90	1.35
SX2	AFAP1L1	-1.59	1.35
SX2	CD101	-1.03	1.35
SX2	DPCD	-0.61	1.35
SX2	FAM40B	-0.67	1.35
SX2	HTRA1	-0.99	1.35
SX2	RGS1	-2.03	1.35
SX3	CENPV	-0.52	6.20
SX3	FN1	-1.14	6.20
SX3	HSPC159	-1.01	6.20
SX3	PGM2L1	-1.02	6.20
SX3	SLC28A3	-1.31	6.20
SX3	UCHL1	-1.80	6.20
SX6	AFAP1L1	-1.59	2.51
SX6	BCAT1	-0.67	2.51
SX6	EPN2	-0.74	2.51
SX6	FNBP1L	-1.67	2.51
SX6	GOLGA7B	-0.77	2.51
SX6	ITGAX	-0.51	2.51
SX6	LPHN1	-0.56	2.51
SX6	LRRFIP1	-0.58	2.51
SX6	PLCXD1	-0.62	2.51
SX6	SLC35F2	-1.13	2.51
SX6	TNFRSF9	-2.11	2.51
SX6	UCHL1	-1.80	2.51

[a]The 3′UTRs of 571 genes selected from the microarray experiment were analyzed by TargetScan [15] and miRanda [16]. For stringency purposes, the targets had to be predicted by both programs to be accepted as a genuine target of the novel miRNA.

[b]Fold changes in down-regulation in mRNA and up-regulation in miRNA in I-Mac compared to M-Mac.

Table 3

Human viruses targeted by novel miRNAs[a]

Novel miRNA	Virus alignment position	Similarity with viral sequence (%)	Viral reference	Viral pathogen targeted
SX1	66169-66188	94.12	NC_001806.1	HSV[b] 1
SX1	68339-68357	100.00	NC_001806.1	HSV 1
SX1	66642-66661	94.12	NC_001798.1	HSV 2
SX1	47189-47209	88.24	NC_001798.1	HSV 2
SX1	110945-110963	93.75	NC_009333.1	HHV[c] 8
SX4	108825-108848	85.71	NC_001806.1	HSV 1
SX5	10577-10602	82.61	NC_001544.1	Ross River virus
SX5	1359-1382	90.00	NC_002058.3	Poliovirus
SX5	12789-12815	83.33	NC_001454.1	Human adenovirus F
SX5	111492-111516	86.36	NC_009334.1	HHV 4
SX5	110662-110686	86.36	NC_009334.1	HHV 4 type 1
SX5	3519-3543	85.71	NC_001355.1	HPV[d] type 6b
SX5	5144-5165	90.00	NC_011800.1	HTLV[e] 4
SX6	5751-5774	81.82	NC_012801.1	Human cosavirus B1
SX6	2590-2615	85.00	NC_014336.1	Human enterovirus 109
SX6	63066-63091	85.71	NC_001806.1	HSV 1
SX6	30823-30845	90.00	NC_001798.1	HSV 2
SX6	121985-122008	95.00	NC_009334.1	HHV 4
SX6	121158-121181	95.00	NC_007605.1	HHV 4 type 1
SX6	118479-118501	100.00	NC_009333.1	HHV 8
SX6	4744-4765	85.00	NC_005134.2	HPV type 96
SX6	4424-4444	85.00	NC_003417.1	Mayaro virus
SX6	154110-154139	70.83	NC_001611.1	Variola virus
SX7	26772-26791	80.95	NC_011203.1	Human adenovirus B1
SX7	48845-48867	85.71	NC_001806.1	HSV 1
SX7	49070-49091	85.71	NC_001798.1	HSV 2
SX7	70373-70397	85.71	NC_001798.1	HSV 2
SX7	147020-147044	90.91	NC_009334.1	HHV 4
SX7	33604-33627	85.7	NC_006273.2	HHV 5
SX7	57193-57216	85.00	NC_009333.1	HHV 8
SX7	2455-2482	84.00	NC_014952.1	HPV type 128

[a]Potential target viral for novel miRNAs were identified by scanning of viral reference sequences from NCBI (ftp://ftp.ncbi.nih.gov/refseq/release/viral/) using miRanda [16].

[b]HSV= herpes simplex virus

[c]HHV = human herpes virus

[d] HPV = human papilloma virus

[e] HTLV = human T-lymphotropic virus