MicroRNA Expression Profile of Spleen CD4$^+$ T Cells from Thioredoxin Primed Lung Transplanted Rats

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Abstract:

We recently reported that priming donor lungs with thioredoxin-1 (Trx) prolongs allograft survival associated with inhibition of host spleen T cell response to donor antigen and cytokine modulation in a rat model of lung transplantation. The molecular events associated with Trx primed allograft survival and modulation of spleen T cell responses remained to be determined. Since micro-RNAs (miRNAs) play a critical role in the regulation of physiological and immunological responses via modulation of target gene and protein expression, we examined the impact of Trx-priming of donor lungs on miRNA expression profile of spleen CD4$^+$T cells isolated from transplanted rats. Donor lungs were primed with Trx for 4 h prior to transplantation. Four days post transplantation recipient spleen CD4$^+$T cells were isolated to identify miRNA profile by microarray analysis using 498 detectable rat miRNAs. Expression of the six mRNA (miR-342-3p, miR-128, miR-30b, miR-92a, miR-25, miR-146a) were significantly up-regulated whereas twelve miRNA (mir-301b, miR-33, miR-210, miR-206, miR-494, miR-29b, miR-196c, miR-362, miR-466b-2, miR-758, miR-466c, miR-101a) were significantly down-regulated in Trx-primed donor lung recipient's spleen CD4+T cells. These sets of miRNAs are associated with modulation of multiple pathways that appeared to play critical role in the regulation of inflammatory responses and T cell proliferation after transplant injury and allograft dysfunction. The impact of Trx-priming on selective miRNA expression with protective role may provide novel therapeutic strategy to limit or prevent injury and promote allograft survival.

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1. Introduction

Lung transplantation is a well-established therapy for patients with end-stage pulmonary diseases (1-3). Survival rate of lung transplant recipient is about 50% at 5 years (3). Despite significant improvements in surgical procedures, organ preservation techniques, and use of antimicrobial and immunosuppressive regimens, acute cellular injury, and lung allograft viability remain a major problem. One of the critical factors involving ischemia/reperfusion (I/R) generated reactive oxygen species (ROS)-mediated acute and chronic injury is causally link with primary graft dysfunction-associated pathogenesis in lung transplantation (4-7). We recently reported that priming rat donor lungs with human recombinant thioredoxin-1 (Trx) prior to transplantation attenuated early I/R injury and progressive infiltration of inflammatory and immune cells that appears to be associated, in part, with scavenging of ROS by Trx (8). Priming of donor lungs prior to transplantation also prevents activation of nuclear factor kappa B (NF-kB), promoted immune tolerance, and prolonged allograft survival in rat model of transplantation (8, 9). Trx, a 12-kDa thiol-disulfide oxidoreductase, is a ROS scavenger and an essential physiological redox regulator of multiple cellular functions including inflammatory and immune responses (10-12). The precise molecular events associated with priming donor lungs with Trx leading to attenuation of inflammatory injury and extended allograft survival remained to be defined.

MicroRNAs (miRNAs) are a class of non-coding small RNAs with approximately 22 nucleotides. The role of miRNAs has been well documented in multiple pulmonary pathophysiologic conditions including in lung allograft rejection (13, 14). They are critical regulators of various cellular processes including gene expression, immunologic responses, and proliferation. Differential expression of miRNAs under inflammatory conditions can also serve as biomarkers of allograft injury. Since priming donor lungs with Trx prior to transplantation prolonged allograft survival by promoting immune tolerance (20), we examined whether Trx-priming impact spleen CD4+T cells miRNA expression prolife in lung transplanted rats.

2. Material and Methods

2.1 Animals

Specific pathogen-free, male Lewis and Sprague-Dawley (SD) rats (250-300 g) obtained from Harlan (Indianapolis, IN) were used in compliance with the Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Institutional Animal Care and Use Committee.

2.2 Surgical Procedures

Lewis donor rat lung were isolated as we previously described (8, 9). The lungs were then flushed with 20 ml preservation solution (Perfadex®, Vitrolife, Uppsala, Sweden) with or without (control) human recombinant purified Trx (4 μg/ml) for 30 min at 4º C. Immediately after flushing the lungs, the tracheostomy tube was clamped following inspiration to preserve the lungs in the inflated state. The heart-lung block was then be removed and placed in ice-cold preservation solution for 4 hr. The left lung was prepared for transplantation with the placement of 14-gauge cuffs into the left main bronchus (MB) and left pulmonary vein (PV) and 16-gauge cuffs into the left PA, respectively.

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2.3 Orthotopic Left Lung Transplantation

Sprague-Dawley recipient animals were anesthetized, intubated and ventilated as described as above. After administration of atropine (0.25 mg/kg) intramuscularly, a left thoracotomy was performed through the fourth intercostal space. The left lung was mobilized by dividing the pulmonary ligament. The hilar structures was then dissected free. The left PA, PV, and MB identified and clamped with microsurgical aneurysm clamps. A ventral incision was made in each of these structures. The cuffs on the donor lung structures were placed into the corresponding recipient structures. The anastomoses were secured with 6.0 silk ties. The transplanted lung was re-inflated. The surgical site was rinsed to assure there was no bleeding and the lung was placed inside the chest cavity. The chest was closed in layers after attaining complete hemostasis. Four days post transplantation, recipient spleens were islolated from control and Trx-primed rats.

2.4 Cell isolation

Recipient's spleen were removed and CD4$^+$ T cells were isolated by positive selection using rat CD4+ isolation kit following instructions from the manufacturer (Miltenyi Biotec, Boston, MA) as previously described (9). The purity of CD4+ T cells was >95%.

2.5 RNA isolation and Quality control

Isolated spleen CD4+ T cells were sent to Ocean Ridge Biosciences (ORB, Deerfield Beach, FL) laboratory for analysis using ORB's proprietary multi-species miRbase version 16 miRNA microarrays. RNA was isolated using TRI reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH), quantified by O.D. measurement and by Quant-iT Ribogreen (Life Technologies, Carlsbad, CA fluorometric assay before quality assessment by gel electrophoresis on a 1% agarose- 2% formaldehyde gel.

The isolated total RNA was digested with RNase-free DNase I (Invitrogen; Carlsbad, CA) and separated into two fractions in order to re-purify digested total RNA (TRD) and low molecular weight (LMW) RNA separately for the different microarray applications.

To re-purify the digested total RNA, RNeasy MinElute columns (Qiagen; Valencia, CA) were used according to manufacturer protocol. To re-purify LMW RNA, 100K Nanosep columns (VWR; Radnor, PA) were used to size fractionate the samples in order to retain low molecular weight RNAs only followed by cleanup using RNeasy MinElute according to the manufacturer protocol. The newly prepared TRD and LMW samples were again quantified by Quant-iT Ribogreen fluorometric assay.

2.6 miRNA Microarray Processing

A total of 50-100 nanograms (ng) of LMW RNA for each sample was 3'-end labeled with Oyster-550 fluorescent dye using the Flash Tag RNA labeling Kit (Genisphere, Hatfield, PA). The labeled RNA samples were hybridized to the microRNA microarrays overnight according to conditions recommended in the Flash Tag RNA labeling Kit manual. The microarrays were scanned on an Axon Genepix 4000B scanner, and data was extracted from images using GenePix V4.1 software.

Spot intensities were obtained for the total number of features on each microarray by subtracting the median local background from the median local foreground for each spot. Visually flagged spots were removed prior to averaging and normalizing. The 95th percentile of the negative control spots

(TPT95) was also calculated for each array. The spot intensities and TPT95 were transformed by taking the log (base 2) of each value. The detection threshold (T) for each microarray was computed by summing five times the standard deviation of the background signal and the 10% trim mean of the negative control spots. The mean probe intensities for each of the probes on each array were then determined by averaging the triplicate spot intensities. The normalization factor (N) for each microarray was determined by obtaining the 20% trim mean of the non-saturated rat probes above threshold in all samples. The log2-transformed spot intensities for all features were normalized, by subtracting N from each spot intensity, and scaled by adding the grand mean of N across all microarrays. Probes with a saturated signal (log2 intensity > 15) in ≥ 90% of samples were removed from the species-specific normalized data set. The non-control, non-saturated, log2-transformed, normalized, and averaged rat probe intensities were filtered to obtain a list of probes showing probe intensity above the normalized thresholds in Trx-primed and control samples.

2.7 Hierarchical Clustering and Principal Component Analysis

Principal Component Analysis (PCA) was performed on the miRNA expression microarray data for all detectable miRNAs within each filtering method. All miRNAs to analyze display using the module built in to the National Institute of Ageing (NIA) Array Analysis software (15). Likewise, the data for the same detectable miRNAs was used for hierarchical clustering analysis by Cluster 3.0 software (16). Hierarchical clustering was conducted using centered correlation as the similarity metric and average linkage as the clustering method. Intensity scale shown is arbitrary.

2.8 Differential Expression Analysis

Statistical analysis of Trx-primed vs Control miRNA expression microarray data set was performed. The log2-transformed and normalized spot intensities for the detectable rat miRNAs were examined for differences between the groups by 1-way ANOVA using National Institute of Ageing (NIA) Array Analysis software (15). This ANOVA was conducted using the Bayesian Error Model, sliding window size of 1000, 10 degrees of freedom and all other settings at their default values. The statistical significance was determined using the False Discovery Rate (FDR) method (18). This represent the proportion of false positives among all miRNAs with P values lower or equal to the P value of the miRNAs that we consider significant. This can also be viewed as an equivalent of a P-value in experiments with multiple hypotheses testing. FDR is an intermediate method between the P-value and Bonferroni correction (multiplying P-value by the total number of probes). The equation is:

$$FDR_r = \min_{i \geq r} \left[\frac{p_i N}{i} \right]$$

where r is the rank of a miRNA ordered by increasing P values, p_i is the P value for probe with rank i, and N is the total number of probes tested. FDR value increases monotonously with increasing P value.

3. Results

3.1 Heat map and cluster analysis

To determine the impact of Trx-priming of donor lungs on spleen CD4+ T cell miRNA expression, we performed cluster analysis of the data using heat map. Fig. 1 shows heat map and cluster analysis of detected miRNAs in control and Trx-primed samples.

The results were analyzed by two-way hirerarchical clustering of miRNAs and samples as described in the methods.

3.2 miRNA expression profile in control and Trx-primed CD4+ T cells

The expression levels of top 30 miRNAs from cluster analysis found to be differentially expressed in Trx-primed and control CD4+ T cell (Table 1). The expression levels of these miRNAs represent >1.5-fold increase or decrease in Trx-primed versus control CD4+ T cells. As shown in Fig. 2, the expression levels of 6 miRNAs (miR-342-3p, miR-128, miR-30b-5p, miR-29a, miR-25, and miR-146a) was significantly increased. Whereas the expression level of 12 miRNAs (miR-301a, miR-33, miR-210, miR-206, miR-494, miR-196c, miR-362, miR-466b-2, miR-758, miR-466c, miR-101a, and miR-29b) was significantly decreased (Fig. 3).

4. Discussion

The results of the present study using a rat model of lung transplantation demonstrate for the first time that priming donor lungs with Trx prior to transplantation significantly alters miRNA expression in spleen CD4+ T cells. This differential expression of miRNAs appears to be associated with multiple molecular events observed in post-transplant allograft injury and rejection including oxidative injury, inflammation, immune response, and fibrosis. Previous reports indicate that Trx-priming of donor lungs reduced acute injury, induced immune tolerance, and extended allograft survival in a rat model of lung transplantation (8, 9). Since selective miRNA expression are known to play a critical role in various pathophysiologic conditions, our observation of Trx-mediated selective expression of miRNA as a biomarker to identify specific cellular response in post-transplant allograft injury/rejection and potentially help develope miRNA based therapeutic strategy.

The differential expression of miRNAs allow to determined association with specific cellular event. We found that significantly up-regulated miRNAs (miR-342-3p, miR-128, miR-30b-5p, miR-29a, miR-25, and miR-146a) or down-regulated miRNAs (miR-301a, miR-33, miR-210, miR-206, miR-494, miR-196c, miR-362, miR-466b-2, miR-758, miR-466c, miR-101a, and miR-29b) appeared to be associated with multiple regulatory functions including inflammation and immune responses. For example, up-regulated miR-146a/b, miR-30a/b, miR-29a have been known to play a critical role in acute injury and inflammation as well as immune responses in multiple organs including lungs (13, 14, 19, 20). Up-regulation of miR-146a is also known to suppress inflammatory responses in in chemical and cytokine-induced acute lung injury (21, 22). Excessive and prolonged IL-1β generation is known to be associated with multiple acute and chronic inflammatory diseases in general (23, 24) and specifically in clinically significant primary graft dysfunction in lung transplant patients (25). Similarly, down-regulation on miRNAs (miR-29 and miR-210) is associated in the modulation of pulmonary fibrosis (26, 27). This is particularly significant in context with Trx-mediated increased expression of miR-146a suggesting suppressive role in acute and/or chronic inflammatory responses or down-regulation of miR-29 and miR-210 in inhibition of fibrosis in post-transplant graft injury and rejection. The potential role of other up-regulated miRNAs (miR-342-3p, miR-128, and miR-25) or down-regulated miRNAs (miR-301a, miR-33, miR-206, miR-494, miR-196c, miR-362, miR-466b-2, miR-758, miR-466c, and miR-101a) in

response to Trx priming and allograft injury remained to be determined.

Priming donor lungs with Trx prior to transplantation offers non-invasive therapeutic strategy to extend allograft survival. Identification of Trx-mediated early biomarkers such as miRNAs is critical for preventing or limiting acute and chronic lung allograft injury and rejection. Our results identified some of the specific miRNAs associated with inflammatory responses and fibrosis. However, more work remained to be determined to establish causal links with miRNAs in the regulation of target genes. Our long-term objectives are to develop miRNA-based therapeutic strategy to prevent early injury and extend lung allograft survival after transplantation.

Acknowledgments

The authors than Ms. Lin Ai and Mr. Bert Herrera for surgery and technical assistant. The study was supported by Gatorade funds #00100966 and by the Department of Veterans Affairs.

Competing Interests

The authors have declared that no competing interests exist.

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Table 1. Impact of Trx-priming on top 30 miRNAs sorted by rank in CD4$^+$T cells

Probe Name	S16_rno_miRNA_name	S16_rno_MIMAT_ID	S16_rno_miRNA_sequence	Source Probe Name	Fold Change 5 vs 4	P	FDR	rank
3197	rno-miR-342-3p	MIMAT0000589	UCUCACACAG	hsa-miR-342-3p	1.82	0.0063	0.9959	1
3054	rno-miR-301a	MIMAT0000552	CAGUGCAAUA	hsa-miR-301a_r	0.47	0.0111	0.9959	2
3114	rno-miR-33	MIMAT0000812	GUGCAUUGUA	hsa-miR-33a_m	0.42	0.0188	0.9959	3
ORB_1210126	rno-miR-210*	MIMAT0017156	AGCCACUGCC	rno-miR-210*	0.58	0.0196	0.9959	4
3240	rno-miR-206	MIMAT0000879	UGGAAUGUAA	hsa-miR-206_m	0.55	0.0239	0.9959	5
ORB_1210610	rno-miR-494	MIMAT0003193	UGAAACAUAC	rno-miR-494	0.58	0.0253	0.9959	6
2757	rno-miR-128	MIMAT0000834	UCACAGUGAA	hsa-miR-128_m	1.58	0.0279	0.9959	7
3145	rno-miR-29b	MIMAT0000801	UAGCACCAUU	hsa-miR-29b_m	0.56	0.0300	0.9959	8
ORB_1210052	rno-miR-196c*	MIMAT0017299	ACAACAACAC	rno-miR-196c*	0.66	0.0345	0.9959	9
2280	rno-miR-30b-5p	MIMAT0000806	UGUAAACAUC	hsa-miR-30b_m	1.52	0.0348	0.9959	10
ORB1275	rno-miR-362*	MIMAT0017357	AACACACCUG	mmu-miR-362-3	0.61	0.0352	0.9959	11
ORB_1210206	rno-miR-466b-2*	MIMAT0017286	AUAUACAUAC	rno-miR-466b-2	0.55	0.0363	0.9959	12
1335	rno-miR-92a	MIMAT0000816	UAUUGCACUU	mmu-miR-92a_r	1.58	0.0382	0.9959	13
ORB_1210670	rno-miR-758*	MIMAT0017316	UGGUUGACCA	mmu-miR-758*	0.64	0.0409	0.9959	14
1139	rno-miR-25	MIMAT0000795	CAUUGCACUU	hsa-miR-25_mn	1.57	0.0413	0.9959	15
ORB_1210545	rno-miR-466c*	MIMAT0017287	UAUACAUGCA	rno-miR-466c*	0.53	0.0435	0.9959	16
2562	rno-miR-101a	MIMAT0000823	UACAGUACUG	hsa-miR-101_m	0.63	0.0473	0.9959	17
1409	rno-miR-146a	MIMAT0000852	UGAGAACUGA	hsa-miR-146a_r	1.65	0.0495	0.9959	18
1313	rno-miR-15b	MIMAT0000784	UAGCAGCACA	hsa-miR-15b_m	1.46	0.0505	0.9959	19
ORB_1210202	rno-miR-466b-1*	MIMAT0017285	AUACAUACAC	rno-miR-466b-1	0.56	0.0519	0.9959	20
1251	rno-miR-30d	MIMAT0000807	UGUAAACAUC	hsa-miR-30d_m	1.54	0.0555	0.9959	21
1416	rno-miR-194	MIMAT0000869	UGUAACAGCA	hsa-miR-194_m	1.47	0.0559	0.9959	22
ORB_0608094	rno-miR-7a-1*	MIMAT0000607	ACAACAAAUC	rno-miR-7a-1*	1.49	0.0585	0.9959	23
ORB_1209050	rno-miR-465	MIMAT0012850	UAUUUAGAAC	rno-miR-465	0.66	0.0616	0.9959	24
1311	rno-miR-195	MIMAT0000870	UAGCAGCACA	hsa-miR-195_m	1.70	0.0649	0.9959	25
ORB_1210546	rno-miR-1949	MIMAT0017852	UAUACCAGGA	rno-miR-1949	0.69	0.0748	0.9959	26
1064	rno-miR-100	MIMAT0000822	AACCCGUAGA	hsa-miR-100_m	1.48	0.0771	0.9959	27
3119	rno-miR-20a	MIMAT0000602	UAAAGUGCUU	hsa-miR-20a_m	1.43	0.0788	0.9959	28
1247	rno-miR-34a	MIMAT0000815	UGGCAGUGUC	hsa-miR-34a_m	0.64	0.0881	0.9959	29
2908	rno-miR-181b	MIMAT0000859	AACAUUCAUU	hsa-miR-181b_r	1.43	0.0894	0.9959	30

The Log2-transformed probe intensities were normalized for all samples as described in methods. Colors red and green indicates >1.5-fold up and down regulation, respectively. Color purple indicate $p< 0.05$.

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Figure Legend

Figure 1. Heat map and clustering results: Spleen CD4+ T cells from Trx-primed and control rats were analysed. The Log2-transformed, normalized, and averaged rat miRNA probes detected in CD4+ T cell using an Axon Genepix 4000B scanner and data was extracted from images using GenePix V4.1 software as described in methods. Red and green indicates >1.5-fold up and down expression, respectively.

Figure 2. Trx-mediated up-regulation of miRNAs. Six miRNAs were found to be significantly ($p<0.05$) up-regulated in spleen CD4+ T cells from Trx-primed samples. The levels of expression are shown in arbitrary units.

Figure 3. Trx-mediated down-regulation of miRNAs. Twelve miRNAs were significantly ($p<0.05$) down regulated in spleen CD4+ T cells from Trx-primed samples. The levels of expression are shown in arbitrary units.

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Figure 1

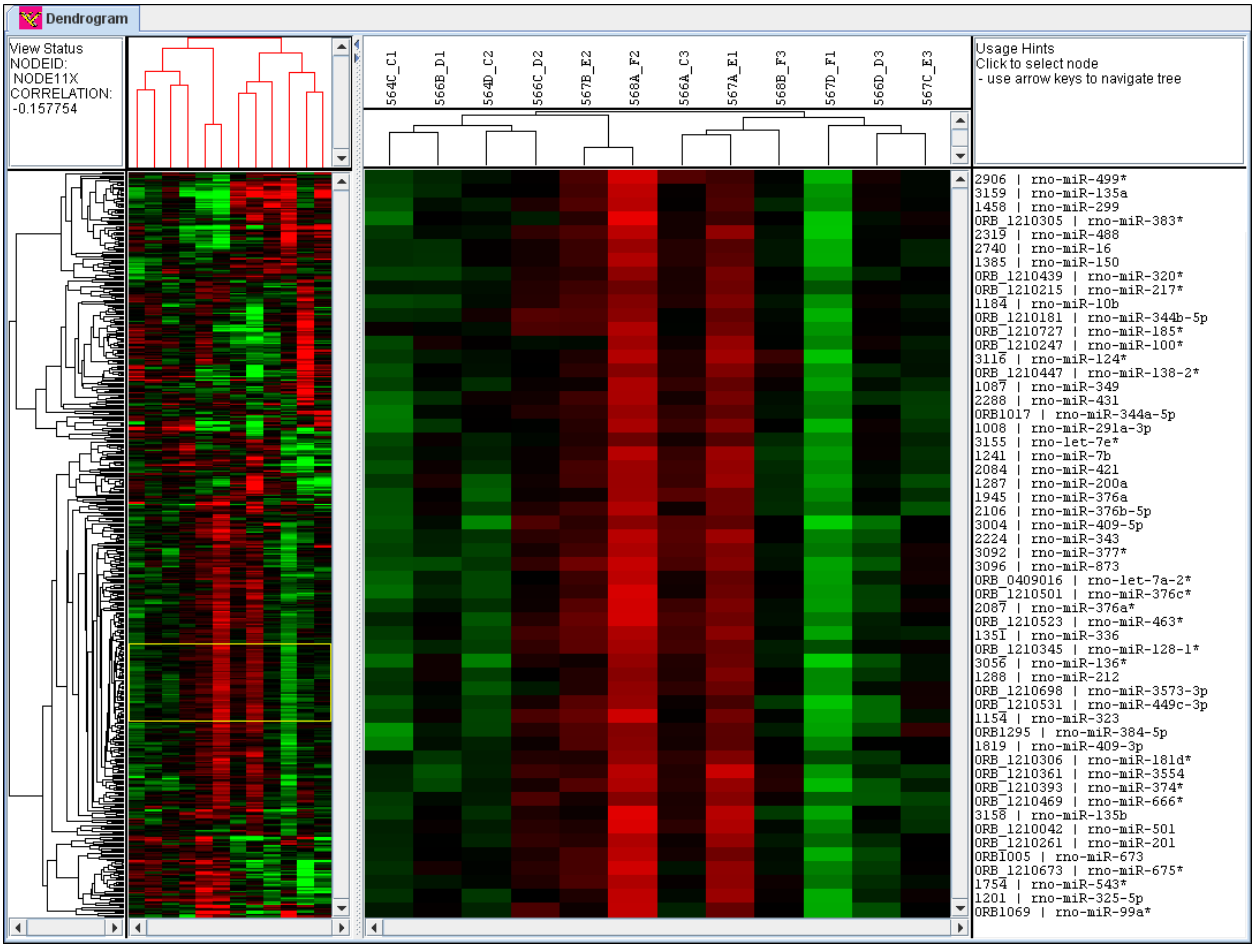

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Figure 2

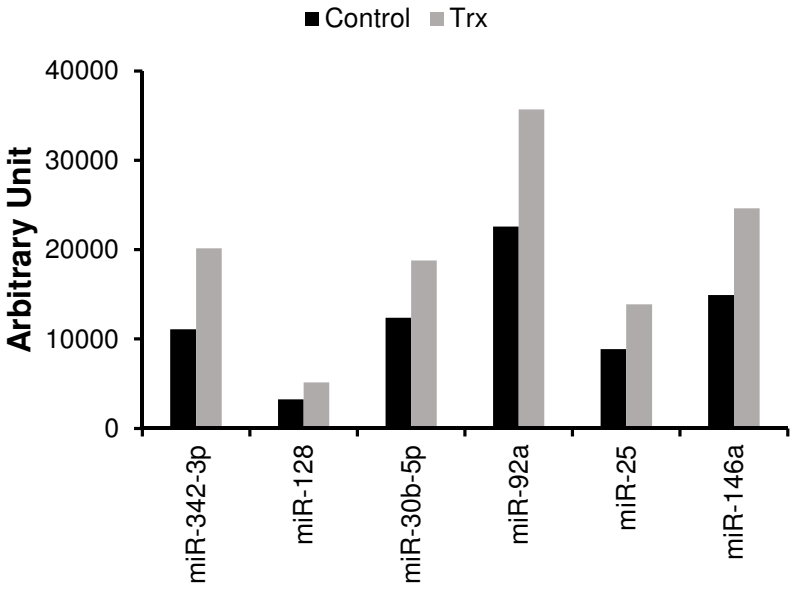

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Figure 3