MESENCHYMAL STEM/STROMAL CELLS INCREASE CARDIAC MIR-187-3P EXPRESSION IN A POLYMICROBIAL ANIMAL MODEL OF SEPSIS

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ABSTRACT—Sepsis-induced myocardial dysfunction (MD) is an important pathophysiological feature of multiorgan failure caused by a dysregulated host response to infection. Patients with MD continue to be managed in intensive care units with limited understanding of the molecular mechanisms controlling disease pathogenesis. Emerging evidences support the use of mesenchymal stem/stromal cell (MSC) therapy for treating critically ill septic patients. Combining this with the known role that microRNAs (miRNAs) play in reversing sepsis-induced myocardial-dysfunction, this study sought to investigate how MSC administration alters miRNA expression in the heart. Mice were randomized to experimental polymicrobial sepsis induced by cecal ligation and puncture (CLP) or sham surgery, treated with either MSCs (2.5×10^5) or placebo (saline). Twenty-eight hours post-intervention, RNA was collected from whole hearts for transcriptomic and microRNA profiling. The top microRNAs differentially regulated in hearts by CLP and MSC administration were used to generate a putative mRNA-miRNA interaction network. Key genes, termed hub genes, within the network were then identified and further validated *in vivo*. Network analysis and RT-qPCR revealed that septic hearts treated with MSCs resulted in upregulation of five miRNAs, including miR-187, and decrease in three top hit putative hub genes (Itpkc, Lrrc59, and Tbl1xr1). Functionally, MSC administration decreased inflammatory and apoptotic pathways, while increasing cardiac-specific structural and functional, gene expression. Taken together, our data suggest that MSC administration regulates host-derived miRNAs production to protect cardiomyocytes from sepsis-induced MD.

KEYWORDS—Gene expression, inflammation, mesenchymal stem/stromal cells, microRNA, mRNA-miRNA network, myocardial dysfunction, sepsis

INTRODUCTION

Myocardial dysfunction (MD) is an important component of multiorgan failure associated with septic shock (1, 2). Septic patients who develop either systolic or diastolic dysfunction have higher mortality, and while the mechanisms remain to be fully elucidated, circulating mediators have been implicated in impaired β-adrenergic signaling, nitric oxide production, altered calcium homeostasis, and metabolic derangement—all contributing to decreased myocardial contractility and left ventricular dilatation (2, 3). Mesenchymal stem cells (MSCs) are multipotent stem/stromal cells reported to confer potent immunosuppressive and immunomodulatory properties (4). In experimental models of sepsis, MSC administration can reduce systemic levels of pro-inflammatory mediators, enhance bacterial clearance, and improve survival (5, 6). Moreover, MSCs have been investigated as an alternative for the treatment for acute myocardial infarction, cardiac remodeling, and cardiovascular disease (7–9). In the context of sepsis, MSCs not only mitigate tissue damage, but also prevent reduction in left ventricular ejection fraction and fractional shortening, indicating that MSCs improve myocardial function (10).

Importantly, since injected MSCs are sequestered in the lung and liver where they remain detectable for only a brief period,

their cardiac protective properties are unlikely to be related to their engraftment and differentiation potential (3, 5, 9). This observation supports the current hypothesis that MSCs dampen the innate immune response of the host in a paracrine fashion during sepsis (9–11). In support of a cell–cell independent therapeutic mechanism, a recent report shows MSC administration mitigates sepsis-induced cardiac dysfunction via inhibition of the mechanistic target of the rapamycin (mTOR) pathway—a ubiquitous serine/threonine kinase that modulates proinflammatory effects and activation status in various cell types—in turn attenuating the expression of inflammation cytokines in cardiac cells (12).

Network analysis of transcriptional changes following MSC administration from our own group supports a role for MSCs in transcriptomic reprogramming involving thousands of genes and multiple pathways in various organs (13). Here, we advanced the work pioneered by our group to demonstrate MSC administration regulates transcriptional networks in a post-transcriptional epigenetic fashion by modulating the expression of cellular micro-RNAs (miRNAs).

MiRNAs are non-coding RNAs of 19–22 nucleotides in length that commonly bind to the 5'- or 3'-UTR of a target mRNA containing a complementary binding sequence called the seed sequence (14). This results in the degradation or destabilization of target mRNAs, and suppression of gene transcription and/or translation—enabling the regulation of hundreds of transcripts in an orchestrated fashion (14–17). Here, we postulate that MSC administration regulates the differential expression of multiple genes simultaneously to generate the transcriptional response profile of the septic heart in response to MSC therapeutic administration. To this end, we have profiled the transcriptome and miRNAome of the murine non-septic and septic heart, treated with placebo or MSCs. Bioinformatic analysis was used to identify top miR-NA:mRNA association pairs. Network analysis identified miR-187–3p and its "hub" gene targets regulated in murine septic hearts in response to MSC administration. In silico prediction of "hub" target gene regulation was validated

in-vivo using an independent polymicrobial sepsis experimental model and in human septic hearts.

MATERIAL AND METHODS

CLP model of sepsis and MSC administration

Archived biobank samples from a previously published experimental sepsis study were used for novel microRNA analysis (4). The original study was approved by the animal care committee at St. Michael's Hospital (Toronto, ON, Canada) in accordance with Canadian Council of Animal Care guidelines. All experiments were conducted in accordance with ARRIVE criteria. Briefly, Mei et al. (4) randomized 8- to 14-week-old male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, Maine) into cecal ligation and puncture (CLP) or sham groups. 6 h post-CLP, mice were further randomized to receive a single intrajugular injection of 2.5×10^5 bone marrow-derived MSCs or placebo (saline) (n = 4). Animals were sacrificed at 28 h and whole hearts were snapped frozen for mRNA and miRNA profiling. A schematic of the experimental model is shown in Figure 1. Transcriptional microarray data have been deposited in GEO [Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo (accession number GSE40180)].

MSC culture

Frozen murine bone marrow-derived MSCs (isolated from male C57Bl/6J mice, courtesy of Dr. Darwin Prockop, Texas A&M Health Science Center) were thawed and cultured according to previously published literature (18). The ability of these MSCs to differentiate into different cell types such as adipocytes, osteocytes, and chondrocytes was previously demonstrated in a published study (19).

Microarray and MicroRNA analysis

Total RNA from whole hearts from four animals per group (sham/saline, CLP/saline, and CLP/MSCs) was extracted and purified. RNA quality was ensured by spectrophotometric analysis (OD260/280). A total of 300 ng of mRNA was hybridized to the Illumina MouseWG-6 v2.0 expression bead array (Illumina Inc, San Diego, Calif). The variance-stabilizing transformation was used to refine normalization using R/Bioconductor software (20). A total of 45,281 probes were selected for analysis. In parallel, 300 ng of total RNA was hybridized on the miRCURY LNA microRNA Array (Exiqon A/S, Vedbaek, Denmark) that queries 2,383 capture microRNA probes. A total of 411 miRNAs whose foreground intensity values were over 1.2 times their background intensity in all samples were chosen for differential analysis (20). Their expression data were then quantile-normalized and batch corrected using the R/Bioconductor packages (20, 21).

Human microarray data (GSE79962, Affymetrix Human Gene 1.0 ST Array) for 24 septic and 11 non-septic hearts was obtained from GEO provided by Matkovitch et al. at Washington University School of Medicine (GEO accession number: GSE79962). We retrospectively analyzed publicly available data that exists on GEO. The raw data for individual chips was normalized and analyzed through Thermofisher's Transcriptome Analysis Console (v 4.0.1).

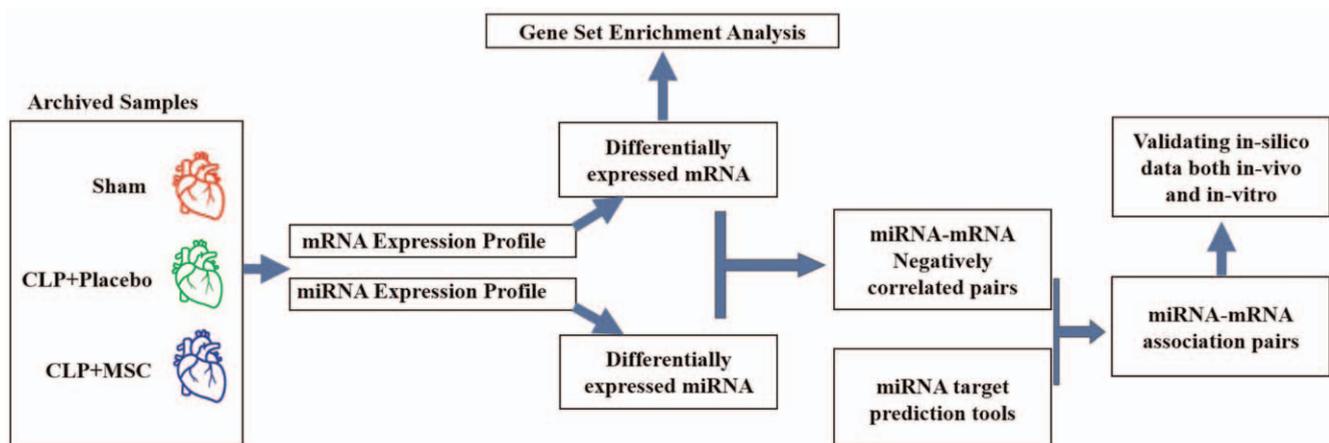Fig. 1. **Experimental design for the *in-vivo* and *in-silico* study leading to identification of miRNA involved in septic heart.** Electronic data (GSE24357) and biobanked samples were analyzed. RNA isolated from archived murine hearts used to generate transcriptomic data (available GSE24357) was profiled using Exiqon arrays. mRNA and microRNA electronic data was then processed to identify differentially associated pairs.

Cardiac myocyte cultures and transfection

Neonatal cardiac myocytes were isolated from the ventricles of 2-day-old control CD1 mice (22). The cells were seeded in F-12 DMEM medium (thermofisher) supplemented with 10% FBS and 5% Pen/Strep (Thermofisher). The next day, the cultures were changed to serum-free medium supplement. Cells were seeded at a concentration of 5×10^5 in 60 mm plates. Media were replaced with antibiotics-free media 12 h before transfection. Cells were transfected with Hiperfect transfection reagent complexed with Qiagen LNA miR-187-3p mimic or mimic negative control (25 nM) according to supplier's instructions for 24 h. The media was then removed and replaced with transfection reagent-free media treated with or without LPS (1 μg/mL) for an additional 24 h. Total RNA was collected from cells using TRIzol (Thermofisher).

Real-time quantitative PCR

Quantitative PCR (qPCR) was used to validate predicted differential expression for the miR-187b and its putative ''hub'' target genes in murine hearts. Total RNA from whole heart tissue was isolated using Tri-Agent (Molecular Research Center Inc) and reverse transcribed to cDNA using the ''SuperScript IV First-Strand Synthesis System'' (Thermofisher). For each hub gene following primers were designed. Sequence can be found in the Supplemental table 1, http://links.lww.com/SHK/B186. The primers for miR-187-5p and miR-187-3p were designed as previously described (Busk, 2014) (23). qPCR was performed with Power SYBR Green Master Mix (Applied Biosystems) using the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). The average threshold count (Ct) value of three technical replicates was used in all calculations. β-actin and U6 were used as loading controls for mRNA and miRNA targets respectively because they displayed the lowest standard deviation among groups compared to other housekeeping genes tested. Data were analyzed using the $2-\Delta Ct$ method depicted by Schmittgen and Livak 2008 (24). Relative mRNA data are expressed as mean ± standard deviation.

Detection of miRNA:mRNA association pairs

The 45,281 Illumina probes were collapsed into 30,869 HUGO gene symbols and ranked by their log-transformed fold change (logFC) values (MSC/CLP) (20, 21). Datasets from the Broad Institute (MSigDB v6.1) and a R/Bioconductor package fgsea version 1.5.2 (minSize=10, maxSize=300, nperm=10,000, padj<0.05) were used to identify overrepresented sets of genes (25, 26). Pathway visualization was done using clusterProfiler package version 3.6.0 (minGSize=10, nPerm=10,000, pvalCutoff=0.05) (27). To find differentially expressed (DE) miRNAs, the R/Bioconductor package limma version 3.34.9 was used (28). Linear regression analysis followed by correction for multiple comparisons (Benjamini-Hochberg adjustment, false discovery rate (FDR) < 0.05) was performed on the miRNA expression data to yield DE miRNAs (28). Next, the Pearson correlation between the expression of 30,869 mRNAs and the DE miRNAs was calculated, and mRNA-miRNA pairs with r < −0.5 and adj. P < 0.05 were selected as negatively correlated pairs. A list of predicted targets for DE miRNAs was obtained from miRanda (score < −0.5, conservation score > 0.5) and TargetScan (context score < −0.3) databases using selected thresholds (29, 30). Based on these data, a list of putative miRNA targets whose expression was negatively correlated with DE miRNAs were selected as high-confident targets (31). Finally, the co-expression state of the high-confident targets was determined by Weighted Gene Co-expression Network Analysis version 1.63 (WGCNA; β = 12, threshold > 0.32) and visualized using Cytoscape software version 3.6.1 (32–34).

RESULTS

MSC administration decreases expression levels of inflammation- and apoptosis-related pathways and increases structural function-related gene sets

Gene set enrichment analysis (GSEA) was used to determine whether *a priori* defined sets of genes are differentially regulated in septic hearts treated with placebo (saline) compared to MSC-treated septic hearts. The MSigDB Hallmark collection was used to perform initial functional enrichment analysis. As previously described by our own group (4), CLP injury significantly decreased the expression of metabolism and bioenergy-related genes. Significant enriched gene sets in MSC-treated septic hearts included genes involved in TNF-α signaling via NF-κB, IL6_JAK_STAT3_signaling, and apoptosis (all FDR≤0.001, nominal P value≤0.01, N = 30,869 gene IDs). In parallel, gene ontology (GO) gene set analysis identified enrichment for key ''GO heart'' related terms in septic hearts treated with MSCs associated with: cardiac conduction, contractile fiber, and regulation of cardiac contraction by calcium ion signaling (Supplemental Table 2, http://links.lww.com/SHK/B187).

We further used GSEA to look at transcriptional profiles in both murine and human septic hearts by aligning gene sets overrepresented in septic versus sham murine hearts with septic versus non-septic in human hearts. Similar to findings in our mice, human sepsis results in increased expression of genes involved in TNF-α Signaling via NF-κB, IL6_JAK_STAT3_-Signaling, and apoptosis in human hearts (FDR ≤0.02 and nominal P values ≤0.012) and decreased expression of oxidative phosphorylation, fatty acid metabolism, and peroxisome activity—consistent with functional homology between human and murine differentially regulated gene sets during sepsis demonstrating the biological relevance of our findings in humans (Supplemental figure 2, http://links.lww.com/SHK/B188). This was also confirmed by upregulation of messenger RNA involved in cardiac muscle structural integrity. Importantly, MSC administration reconstituted the expression (back to sham-like levels) of over 60% of genes whose expression levels were decreased in CLP mice treated with placebo (Fig. 2).
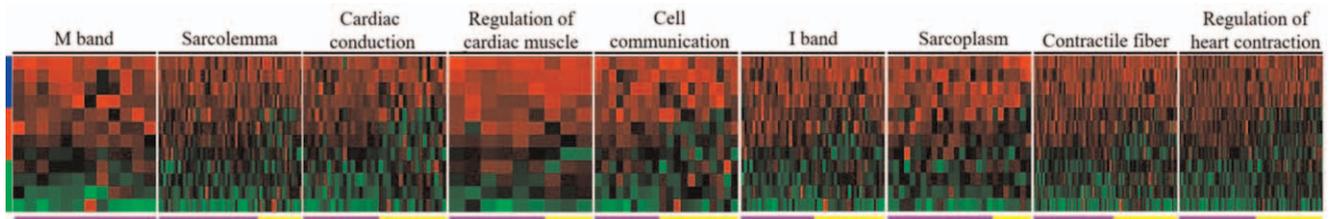
FIG. 2. **Administration of MSCs into Sham or CLP animals causes differential expression of genes involved in heart function.** Heat Map showing relative expression of genes (rows) encoding for proteins involved in M Band, Sarcolemma, Cardiac Conduction, Regulation of Cardiac Muscle Contraction by Calcium Ion Signaling, Cell Communication Involved in Cardiac Conduction, I Band, Sarcoplasm, Contractile Fiber, Regulation of Heart Contraction. Green, red, and blue bars indicate CLP, MSC, and Sham mice, respectively. Purple bar indicates upregulated genes after MSC treatment, while yellow indicates genes remained downregulated. By convention, red indicates upregulation and green downregulation. Samples (columns) are clustered using the complete linkage method. CLP indicates cecal ligation and puncture; MSC, mesenchymal stem cell.

MSC administration decreased heart expression of inflammatory-mediator-related receptor gene sets

Canonical pathways such as the KEGG cytokine-cytokine receptor pathway were significantly downregulated (NES = −1.86, adjusted P value = 0.00566) in MSC- versus placebo-treated CLP mice. Although mRNA for a few cytokine ligands such as CXCL1, CXCL2, and IL-19 were significantly decreased, the most pronounced effect of MSC administration in CLP murine hearts involved the reconstitution of receptor expression levels back to sham. Important receptor mRNAs affected included: CCR6, CCR1, IL6R, OSMR, IL13RA1, CSF2RB, EGFR, IL20RB, SF12A, SF1A, LTBR, SF18, ACVR1B, IL1R2, and IL18R1 (Supplemental Figure 1, http://links.lww.com/SHK/B189). Taken together, our data suggest MSCs modulate the response to specific pro-inflammatory stimuli in the heart by primarily modulating the expression levels of their specific receptors.

MSC administration altered expression of cellular microRNAs

A total of 411 miRNAs that passed quality control and were deemed to be expressed in murine hearts were included in the differential expression analysis using LIMMA. Two main comparisons were made: Sham versus CLP treated with placebo (saline, CLP+placbo) and CLP+placebo versus CLP treated with MSCs (CLP+MSC). The result showed that four miRNAs were upregulated, and 11 miRNAs were downregulated after CLP surgery (Table 1 and Fig. 3A). In response to MSC treatment, five miRNAs were upregulated, and none were downregulated in septic hearts compared to placebo (Table 2).

Network analysis and identification of miR-187b-3p "hub" genes

To elucidate the putative function of the five miRNAs identified as differentially expressed in MSC versus CLP, miRNA:mRNA pairs with strong negative correlation (r <

−0.5, $P = 0.05$), and predicted target interactions from miRanda (score < −0.5, conservation score > 0.5) and TargetScan (context score < −0.3) were selected for further analysis (Fig. 3B). The overlapping 341 miRNA:mRNA pairs, or the interaction of total 318 mRNAs and five miRNAs, were obtained as highly confident interactions based on transcriptional profiles from septic MSC- and placebo-treated murine hearts (Fig. 2). We used weighted gene correlation network analysis (WGCNA) to identify among the 318 genes a total of 53 highly co-expressed genes (β = 12, threshold > 0.32) and mapped these using five different colors indicating relationships between the gene targets and each of their putative regulatory miRNAs (Fig. 3). Hub genes, highly interconnected genes, were identified: Inositol-Trisphosphate 3-Kinase C (Itpkc), Leucine-Rich Repeat-Containing Protein 59 (Lrrc59), Transducin Beta Like 1 X-Linked Receptor 1 (Tbl1xr1), and Strawberry Notch Homolog 2 (Sbno2). Three of these were targets of miR-187, and one was the target of miR-2139 (Fig. 4).

Validation of miR-187-3p genes in-vitro and in-vivo

To validate the in-silico hypothesis, we elected to demonstrate differential regulation of miR-187-3p, Itpkc, Lrrc59, and Tbl1xr1 in independent experiments. First, we identified a total of 161 miR-187 targets and determined differential regulation of these targets in both our murine transcriptomics data from septic hearts by comparing expression profiles in sham versus CLP+placebo-treated hearts. We then compared differential regulation of the miR-187-3p gene set in human hearts to show similar biology occurs in human septic hearts compared to non-septic hearts. Metascape was used to perform functional enrichment for these 161 genes shown to be regulated in human and murine hearts by sepsis revealing involvement in critical pathways such as IL-6 production, IL-1 family signaling, and antigen presentation (Fig. 5B). Using IntaRNA RNA-RNA interaction prediction tool we were able to demonstrate that

TABLE 1. **MSC treatment reverses the expression of downregulated miRNA in septic heart of CLP mice**

miRNA	Fold change			Adjusted P value		
	MSC/CLP	MSC/Sham	CLP/Sham	MSC/CLP	MSC/Sham	CLP/Sham
mmu-miR-470	1.600	1.176	0.735	0.0379	>0.05	>0.05
mmu-miR-125b-1-3p	1.116	1.041	0.933	0.0379	>0.05	>0.05
mmu-miR-187	1.328	0.955	0.719	0.0141	>0.05	0.00599
mmu-miR-2137	1.629	0.653	0.401	0.0271	>0.05	3.20E-05
mmu-miR-2139	1.148	0.986	0.859	0.0475	>0.05	0.0269
mmu-miR-290-5p	0.971	0.573	0.590	>0.05	0.0379	>0.05
mmu-miR-762	1.105	0.628	0.568	>0.05	0.0141	0.00383
mmu-miR-3090	0.977	0.660	0.676	>0.05	0.00824	0.00986
mmu-miR-3102	0.948	0.653	0.689	>0.05	0.00383	0.00986
mmu-miR-29b-1	1.031	0.888	0.862	>0.05	>0.05	0.0475
mmu-miR-30a	0.924	1.108	1.198	>0.05	>0.05	0.0432
mmu-miR-711	1.216	0.915	0.752	>0.05	>0.05	0.00986
mmu-miR-883a-5p	1.319	0.913	0.692	>0.05	>0.05	0.0379
mmu-miR-294	1.247	0.881	0.707	>0.05	>0.05	0.0103
mmu-miR-193b	1.200	0.958	0.798	>0.05	>0.05	0.0186
mmu-miR-1195	0.922	1.031	1.118	>0.05	>0.05	0.0342
mmu-miR-30e	0.888	1.205	1.357	>0.05	>0.05	0.0156
mmu-miR-208a-5p	0.830	1.083	1.306	>0.05	>0.05	0.00986

Fold change and adjusted P value (Benjamini−Hochberg) of multiple comparisons between MSC/CLP, MSC/Sham, and CLP/Sham represent changes in miRNA expression between treatments. $P < 0.05$ was established as the threshold for selecting DE miRNA.
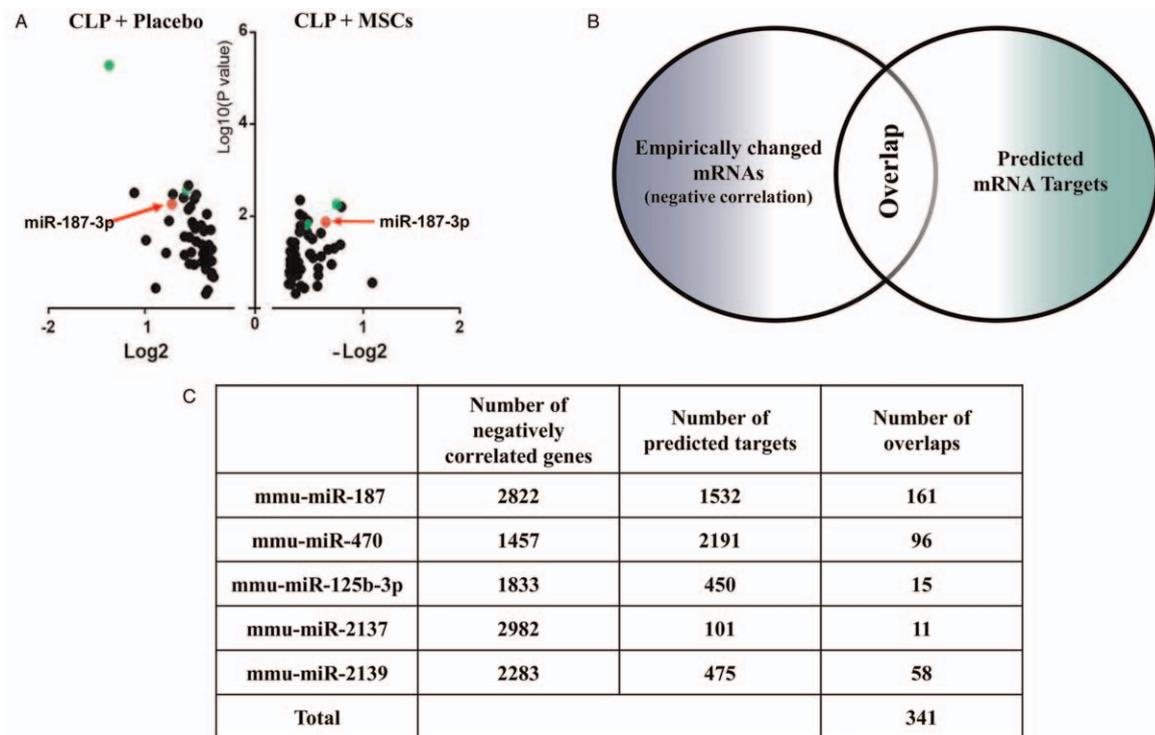
Fig. 3. **Detection of highly confident gene sets.** A, Volcano plot of DE miRNA versus Fold change, red is miR-187 and green dots represent miR-2139 and miR-2137. B, Venn diagram defining highly confident gene sets. C, Table showing the overlap between number of predicted gene targets for DE miRNAs and the number of negatively correlated genes.

there are high affinity binding sites between miR-187-3p seed sequence and the hub genes, supporting the hypothesis that miR-187 binds and regulates the expression of those hub genes in heart. The three hub genes ITPKC, LRRC59, and TBL1 were consistently differentially expressed between septic and healthy hearts in both human and mouse.

In-vivo and in-vitro validation of miR-187-3p and its putative "hub" gene targets regulation by MSCs in septic hearts

We confirmed differential regulation of ITPKC, LRRC59, and TBL1 during experimental sepsis and in response to MSC administration in independent *in-vivo* experiments by semi-quantitative RT-PCR (Fig. 6).

In gain of function experiments, we transfected primary neonatal cardiomyocytes with exogenous miR-187-3p mimic and then treated the cells with LPS. Results show that transfection of primary cardiomyocytes with the miR187-3p resulted in decreased expression of ITPKC, LRRC59, and TBL1 in LPS-treated cardiomyocytes further aligned with in-silico data, validating miR-187 targeting these genes (Fig. 7).

DISCUSSION

Using the Illumina expression array, we measured the expression of 30,869 genes in whole murine hearts harvested at 28 h post induction of polymicrobial sepsis. As previously

TABLE 2. **Summary table of significantly differentially expressed miRNAs in the heart of CLP animals**

	Upregulated in sepsis	Downregulated in sepsis	Expression not affected by sepsis
Upregulated by MSC treatment	None	mmu-miR-187 mmu-miR-2137 mmu-miR-2139	mmu-miR-470 mmu-miR-125b-3p
Downregulated by MSC treatment	None	None	None
Not affected by MSC treatment	mmu-miR30a mmu-miR1195 mmu-miR-30e mmu-miR208-5p	mmu-miR-762 mmu-miR-3090 mmu-miR-3102 mmu-miR-29b-1 mmu-miR-883a-5p mmu-miR-294 mmu-miR-193b	mmu-miR-394

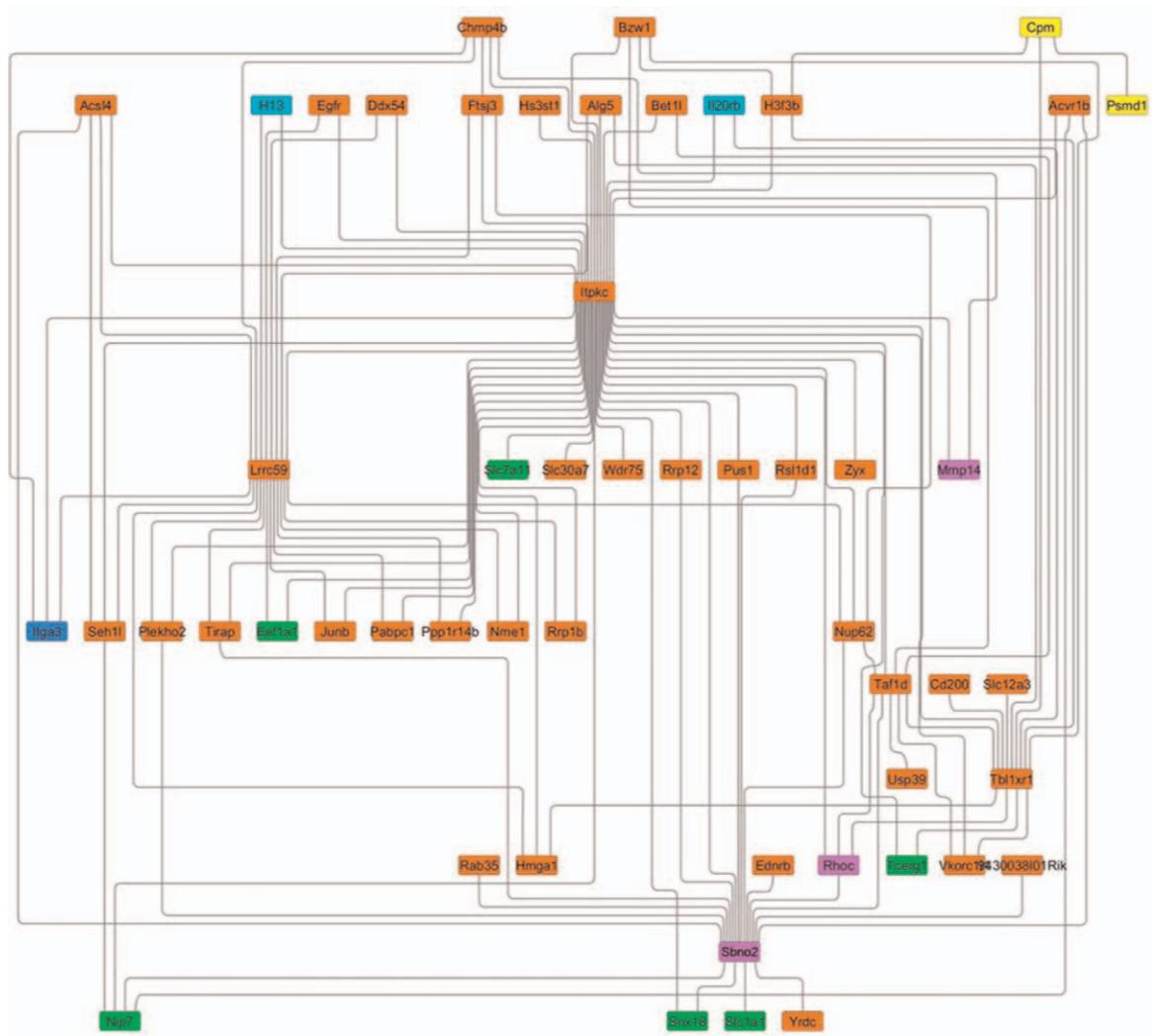Adj. $P < 0.05$ was established as the threshold for selecting DE miRNA.

FIG. 4.   **Co-expression network of DE miRNA targets.** Hub genes are highlighted in Orange: miR-187 targets, Yellow: miR-2139, Cyan: miR-125b-1-3p, Green: miR-470, and Purple: miR-2137. Merged colors in a node indicating the gene is targeted by multiple miRNAs.

reported (3–7, 18), MSC treatment attenuated inflammation and apoptosis. The RNA microarray results also suggest that of the 485 genes involved in nine gene ontology terms associated with cardiac functions, 39% (191/485) were downregulated following CLP of which 64% (122/191) were subsequently altered by MSC treatment. This suggests that MSC administration returns gene transcription to non-septic levels (i.e., similar to sham mice), supporting current knowledge that MSC treatment is cardio-protective in sepsis (5, 17). Our data further supports previously published data from our group showing "pan-organ" analysis of the transcriptomic profile following MSC administration (13).

The Exiqon expression array identified 15 sepsis-related differentially expressed miRNAs and five MSC-related differentially expressed miRNAs in CLP heart treated with placebo versus MSC respectively. Biological plausibility was established by functional enrichment analysis showing that various gene products are known to be relevant to sepsis (34, 35). Of the miRNAs found to be regulated following MSC administration, all were upregulated compared to the placebo-treated CLP group. Three of them, miR-187, miR-2137, and miR-2139,

were also downregulated in septic hearts (CLP-placebo). Since the expression of these three miRNAs is not statistically significantly different in the MSC-treated versus the sham group, it is possible MSC administration may have "rescued" the expression of these three miRNAs—reconstituting normal expression profiles associated with improved outcomes in the MSC-treated group. In contrast, miR-470 and miR-125b-1-3p were not altered following CLP—suggesting that while these microRNAs may be altered in sepsis, they may not play a therapeutic role following MSC administration.

Gene regulatory networks are generally thought to be made up of a few highly connected nodes (hubs) and many poorly connected nodes nested within a hierarchical regulatory regime. This is consistent with the view that most genes have limited pleiotropy and operate within regulatory modules (36). The miRNA:mRNA combined network analysis revealed that Itpkc, Lrrc59, Tbl1xr1, and Sbno2 are "hub" genes (highly connected intramodular genes) that may play critical roles in MSC-mediated regulation and cardio-protection. All four hub genes identified are mouse orthologs of human ITPKC, LRRC59, TBL1XR1, and SBNO2.
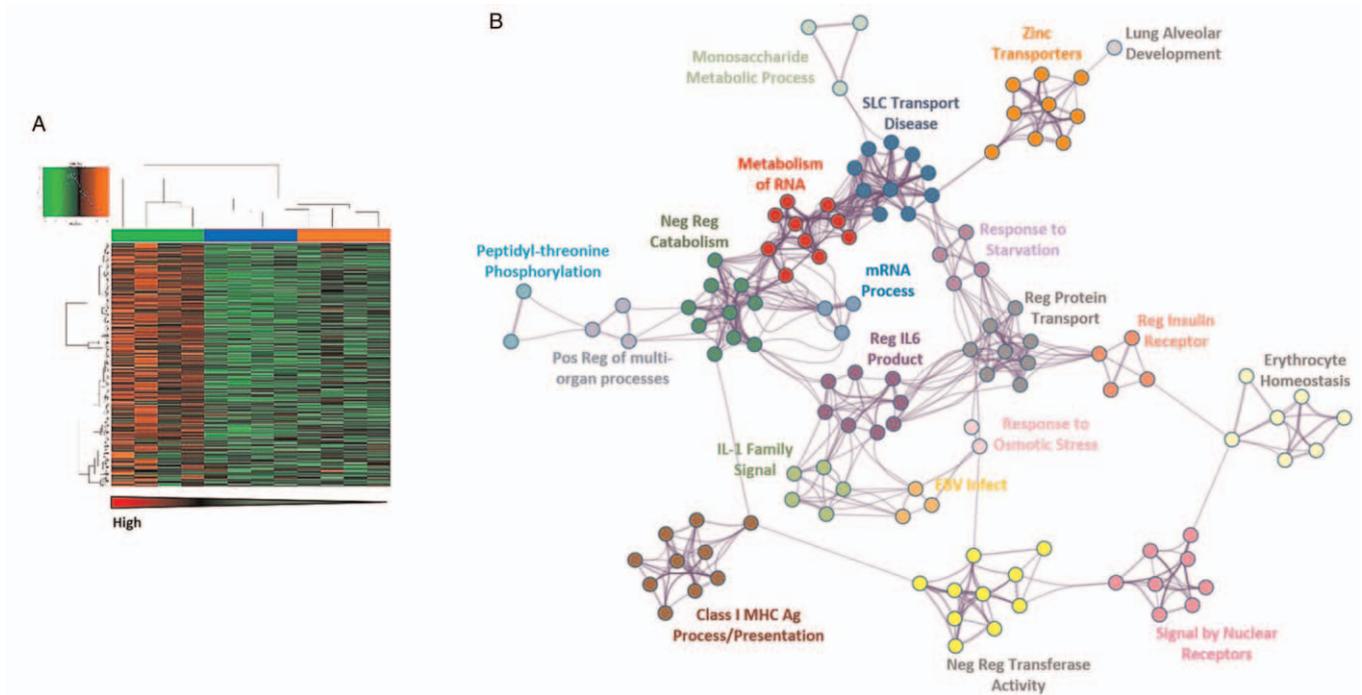
Fig. 5. **MSC-driven miR-187 increased expression regulates multiple targets in septic heart.** A, Heat Map showing Robust multichip average (RMA) normalized expression values of highly confident genes (rows). Green, blue, and red bars indicate CLP, Sham, and MSC mice, respectively (columns). By convention red represents upregulation and green downregulation. Genes and samples were clustered using the complete linkage method. List of targets can be found in Supplemental table 3, http://links.lww.com/SHK/B190. B, Metascape plot showing functional enrichment for 161 genes shown to be regulated in human and murine hearts by sepsis.

A literature review identified four of the hub genes as involved in the inflammatory response. Itpkc (inositol 1,4,5-triphosphate kinase C) is a kinase that phosphorylates IP3 to prevent its binding to the IP3 receptor on the endoplasmic/sarcoplasmic reticulum. This phosphorylation prevents calcium influx to the cytosol, downregulating the Ca^{2+}/NFAT pathway and calcium-dependent inflammation and apoptosis (37). Defects in ITPKC are reported in patients of Kawasaki disease, in which dysregulated T-cells attack the vasculature to cause vasculitis and coronary artery aneurysm (38, 39). This inflammation is linked to the upregulation of NLRP3 in response to an increase in cytosolic calcium (39). However, it has been argued that NLRP3 upregulation in the cardiac tissue is possibly more cardio-protective than pro-inflammatory or pro-apoptotic due

to its potential to activate the reperfusion injury salvage kinase (RISK) pathway (40). Therefore, a reduction in Itpkc in the cardiac tissue, as observed in our MSC groups, may be host protective.

Lrrc59 (leucine-rich repeat containing protein 59) is a type II transmembrane protein that plays a role in nuclear transport at the nuclear membrane as well as COPII-coated vesicle trafficking from the endoplasmic reticulum (41, 42). Lrrc59 regulates the endosomal localization of nucleic acid-sensing toll-like receptors (TLRs) such as TLR3, 7, 8, and 9, specifically ligand-dependent trafficking of TLRs from the ER to endosomes/lysosomes so that knockdown of Lrrc59 reduces TLR trafficking and signaling, implying the downregulation of Lrrc59 may have a fundamental anti-inflammatory role (42).
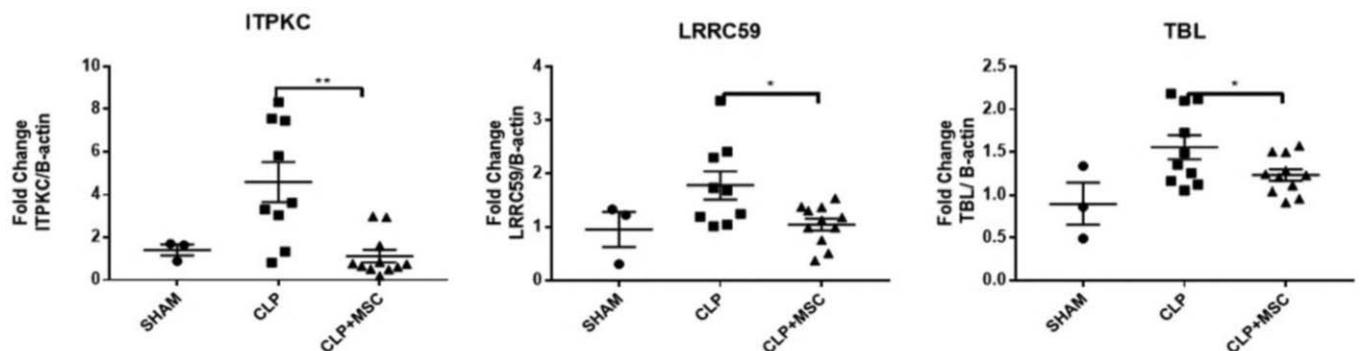

Fig. 6. **MSC treatment lowers expression of miR-187 targets in septic heart.** The expression of miR-187 targets; ITPKC, LRRC59, and TBL1 are determined using quantitative polymerase chain reaction. Error bars represent standard error of the mean and * represents significant ($P < 0.05$), ** represent significant ($P < 0.01$).
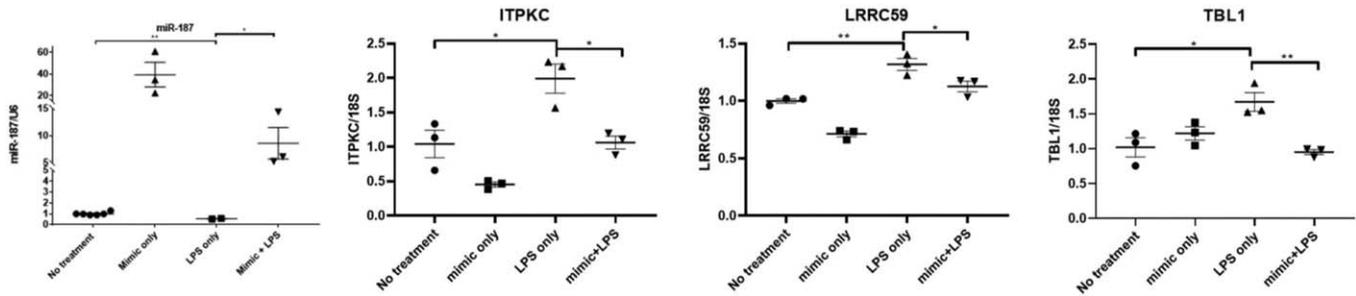
Fig. 7. **miR-187 mimic lowers expression of miR-187 targets in LPS-treated myocytes.** The expression of miR-187 targets; ITPKC, LRRC59, and TBL1 are determined using quantitative polymerase chain reaction. Error bars represent standard error of the mean and *represents significant ($P < 0.05$), **represent significant ($P < 0.01$).

Tbl1xr1 (transducin β-like 1 X-linked receptor 1) is essential in the activation of Wnt-β-catenin and NF-κB signaling pathways. This protein is expressed in the nucleus and is critical for the recruitment of β-catenin and NF-κB to Wnt target genes and NF-κB target genes, respectively (43, 44). The suppression of Tbl1xr1 may explain the reduction in inflammation observed in MSC-treated mice. These three genes are putative targets of miR-187, based on the miRanda and TargetScan data (45). It has been previously reported that miR-187 negatively regulates TNF-α, IL-6, and IL-12p40 transcription, and works as an anti-inflammatory miRNA (46). The detailed mechanism of this regulation is not fully elucidated, but our results suggest that—in the septic heart—this modulation is potentially achieved by reduction in expression levels of Itpkc, Lrrc59, and Tbl1xr1.

Based on these findings, we postulate that improved outcomes in MSC-treated mice are partially the result of miR-187 differential expression. MSC administration results in differential expression of miR-187 in recipient hearts. miR-187 in turn exerts its post-transcriptional regulatory effect by targeting hub genes which play role in anti-inflammatory pathways. This miRNA destabilizes the mRNAs of immune regulators such as Itpkc, Lrrc59, and Tbl1xr1. It is worth noting that the underlying function of miR-187 in cardiomyocytes and the heart at large requires further investigation. We hypothesize miR-187-induced regulation of immune receptors and inflammatory pathways mitigates the dysregulated inflammatory response of the heart to mediate MSC-dependent cardio-protective effects, which may improve survival in septic mice treated with MSCs.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, et al.: The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 315(8):801–810, 2016.
2. Drosatos K, Lymperopoulos A, Kennel PJ, Pollak N, Schulze PC, Goldberg IJ: Pathophysiology of sepsis-related cardiac dysfunction: driven by inflammation, energy mismanagement, or both? *Curr Heart Fail Rep* 12(2):130–140, 2015.
3. Németh K, Leelahavanichkul A, Yuen PS, Mayer B, Parmelee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, et al.: Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 15(1):42–49, 2009.
4. Mei SH, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart DJ: Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 182(8):1047–1057, 2010.
5. Weil BR, Herrmann JL, Abarbanell AM, Manukyan MC, Poynter JA, Meldrum DR: Intravenous infusion of mesenchymal stem cells is associated with improved myocardial function during endotoxemia. *Shock* 36(3):235–241, 2011.
6. Anderson P, Souza-Moreira L, Morell M, Caro M, O'Valle F, Gonzalez-Rey E, Delgado M: Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut* 62(8):1131–1141, 2013.
7. Grove JE, Bruscia E, Krause DS: Plasticity of bone marrow-derived stem cells. *Stem Cells* 22(4):487–500, 2004.
8. Suzuki E, Fujita D, Takahashi M, Oba S, Nishimatsu H: Therapeutic effects of mesenchymal stem cell-derived exosomes in cardiovascular disease. *Adv Exp Med Biol* 998:179–185, 2017.
9. Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 276(5309):71–74, 1997.
10. Müller-Ehmsen J, Krausgrill B, Burst V, Schenk K, Neisen UC, Fries JW, Fleischmann BK, Hescheler J, Schwinger RH: Effective engraftment but poor mid-term persistence of mononuclear and mesenchymal bone marrow cells in acute and chronic rat myocardial infarction. *J Mol Cell Cardiol* 41:876–884, 2006.
11. Loboda A, Stachurska A, Florczyk U, Rudnicka D, Jazwa A, Wegrzyn J, Kozakowska M, Stalinska K, Poellinger L, Levonen AL, et al.: HIF-1 induction attenuates Nrf2-dependent IL-8 expression in human endothelial cells. *Antioxid Redox Signal* 11:1501–1517, 2009.
12. Huang W, Fan W, Wang Y, Han D, Li X, Li S, Li C, Xu B, Huang Y, Fu X, et al.: Mesenchymal stem cells in alleviating sepsis-induced mice cardiac dysfunction via inhibition of mTORC1-p70S6K signal pathway. *Cell Death Discov* 3:16097, 2017.
13. dos Santos CC, Murthy S, Hu P, Shan Y, Haitsma JJ, Mei SH, Stewart DJ, Liles WC: Network analysis of transcriptional responses induced by mesenchymal stem cell treatment of experimental sepsis. *Am J Pathol* 181(5):1681–1692, 2012.
14. Collino F, Bruno S, Deregibus MC, Tetta C, Camussi G: MicroRNAs and mesenchymal stem cells. *Vitam Horm* 87:291–320, 2011.
15. Gu W, Xu Y, Xie X, Wang T, Ko JH, Zhou T: The role of RNA structure at 5' untranslated region in microRNA-mediated gene regulation. *RNA* 20(9):1369–1375, 2014.
16. Miao L, Yao H, Li C, Pu M, Yao X, Yang H, Qi X, Ren J, Wang Y: A dual inhibition: microRNA-552 suppresses both transcription and translation of cytochrome P450 2E1. *Biochim Biophys Acta* 1859(4):650–662, 2016.
17. Wang X, Gu H, Qin D, Yang L, Huang W, Essandoh K, Wang Y, Caldwell CC, Peng T, Zingarelli B, et al.: Exosomal miR-223 contributes to mesenchymal stem cell-elicited cardioprotection in polymicrobial sepsis. *Sci Rep* 5:13721, 2015.
18. Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ: Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood* 103:1662–1668, 2004.
19. Mei SH, McCarter SD, Deng Y, Parker CH, Liles WC, Stewart DJ: Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 4(9):e269, 2007.
20. Du P, Kibbe WA, Lin SM: lumi: a pipeline for processing Illumina microarray. *Bioinformatics* 24(13):1547–1548, 2008.
21. Brock GN, Mukhopadhyay P, Pihur V, Webb C, Greene RM, Pisano MM: MmPalateMiRNA, an R package compendium illustrating analysis of miRNA microarray data. *Source Code Biol Med* 8(1):1, 2013.
22. Tsoporis JN, Izhar S, Proteau G, Slaughter G, Parker TG: S100B-RAGE dependent VEGF secretion by cardiac myocytes induces myofibroblast proliferation. *J Mol Cell Cardiol* 52(2):464–473, 2012.

23. Busk PK: A tool for design of primers for microRNA-specific quantitative RT-qPCR. *BMC Bioinformatics* 15:29, 2014.

24. Schmittgen TD, Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3(6):1101–1108, 2008.

25. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD: The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 28(6):882–883, 2012.

26. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, et al.: Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102(43):15545–15550, 2005.

27. Powers RK, Goodspeed A, Pielke-Lombardo H, Tan AC, Costello JC: GSEA-InContext: identifying novel and common patterns in expression experiments. *Bioinformatics* 34:i555–i564, 2018.

28. Yu G, Wang LG, Han Y, He QY: clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16(5):284–287, 2012.

29. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3:Article3, 2004.

30. Betel D, Koppal A, Agius P, Sander C, Leslie C: Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol* 11(8):R90, 2010.

31. Agarwal V, Bell GW, Nam JW, Bartel DP: Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 4:e05005, 2015.

32. Lourdusamy A, Rahman R, Smith S, Grundy R: microRNA network analysis identifies miR-29 cluster as key regulator of LAMA2 in ependymoma. *Acta Neuropathol Commun* 3:26, 2015.

33. Zhang B, Horvath S: A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol* 4:17, 2005.

34. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T: Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13(11):2498–2504, 2003.

35. Wang HJ, Zhang PJ, Chen WJ, Feng D, Jia YH, Xie LX: Four serum microRNAs identified as diagnostic biomarkers of sepsis. *J Trauma Acute Care Surg* 73(4):850–854, 2012.

36. Caserta S, Mengozzi M, Kern F, Newbury SF, Ghezzi P, Llewelyn MJ: Severity of systemic inflammatory response syndrome affects the blood levels of circulating inflammatory-relevant MicroRNAs. *Front Immunol* 8:1977, 2018.

37. van Dam S, Võsa U, van der Graaf A, Franke L, de Magalhães JP: Gene co-expression analysis for functional classification and gene-disease predictions. *Brief Bioinform* 19(4):575–592, 2017.

38. Yeter D, Deth R: ITPKC susceptibility in Kawasaki syndrome as a sensitizing factor for autoimmunity and coronary arterial wall relaxation induced by thimerosal's effects on calcium signaling via IP3. *Autoimmun Rev* 11(12):903–908, 2012.

39. Alphonse MP, Duong TT, Shumitzu C, Hoang TL, McCrindle BW, Franco A, Schurmans S, Philpott DJ, Hibberd ML, Burns J, et al.: Inositol-Triphosphate 3-Kinase C mediates inflammasome activation and treatment response in Kawasaki disease. *J Immunol* 197(9):3481–3489, 2016.

40. Sandanger Ø, Gao E, Ranheim T, Bliksøen M, Kaasbøll OJ, Alfsnes K, Nymo SH, Rashidi A, Ohm IK, Attramadal H, et al.: NLRP3 inflammasome activation during myocardial ischemia reperfusion is cardioprotective. *Biochem Biophys Res Commun* 469(4):1012–1020, 2016.

41. Zhen Y, Sørensen V, Skjerpen CS, Haugsten EM, Jin Y, Wälchli S, Olsnes S, Wiedlocha A: Nuclear import of exogenous FGF1 requires the ER-protein LRRC59 and the importins Kpnα1 and Kpnβ1. *Traffic* 13(5):650–664, 2012.

42. Tatematsu M, Funami K, Ishii N, Seya T, Obuse C, Matsumoto M: LRRC59 regulates trafficking of nucleic acid-sensing TLRs from the endoplasmic reticulum via association with UNC93B1. *J Immunol* 195(10):4933–4942, 2015.

43. Li J, Wang CY: TBL1-TBLR1 and beta-catenin recruit each other to Wnt target-gene promoter for transcription activation and oncogenesis. *Nat Cell Biol* 10(2):160–169, 2008.

44. Hoberg JE, Yeung F, Mayo MW: SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival. *Mol Cell* 16(2):245–255, 2004.

45. Rossato M, Curtale G, Tamassia N, Castellucci M, Mori L, Gasperini S, Mariotti B, De Luca M, Mirolo M, Cassatella MA, et al.: IL-10-induced microRNA-187 negatively regulates TNF-α, IL-6, and IL-12p40 production in TLR4-stimulated monocytes. *Proc Natl Acad Sci U S A* 109(45):E3101–E3110, 2012.

46. El Kasmi KC, Smith AM, Williams L, Neale G, Panopoulos AD, Watowich SS, Häcker H, Foxwell BM, Murray PJ: Cutting edge: a transcriptional repressor and corepressor induced by the STAT3-regulated anti-inflammatory signaling pathway. *J Immunol* 179(11):7215–7219, 2007.