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Transcriptomic Profile of Lin⁻Sca1⁺c-kit (LSK) cells in db/db mice with long-standing diabetes

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Abstract

Background The Lin⁻Sca1⁺c-Kit⁺ (LSK) fraction of the bone marrow (BM) comprises multipotent hematopoietic stem cells (HSCs), which are vital to tissue homeostasis and vascular repair. While diabetes affects HSC homeostasis overall, the molecular signature of mRNA and miRNA transcriptomic under the conditions of long-standing type 2 diabetes (T2D;>6 months) remains unexplored.

Methods In this study, we assessed the transcriptomic signature of HSCs in db/db mice, a well-known and widely used model for T2D. LSK cells of db/db mice enriched using a cell sorter were subjected to paired-end mRNA and single-end miRNA seq library and sequenced on Illumina NovaSeq 6000. The mRNA sequence reads were mapped using STAR (Spliced Transcripts Alignment to a Reference), and the miRNA sequence reads were mapped to the designated reference genome using the Qiagen GeneGlobe RNA-seq Analysis Portal with default parameters for miRNA.

Results We uncovered 2076 out of 13,708 mRNAs and 35 out of 191 miRNAs that were expressed significantly in db/db animals; strikingly, previously unreported miRNAs (miR-3968 and miR-1971) were found to be downregulated in db/db mice. Furthermore, we observed a molecular shift in the transcriptome of HSCs of diabetes with an increase in pro-inflammatory cytokines (*Il4*, *Tlr4*, and *Tnf11a*) and a decrease in anti-inflammatory cytokine *IL10*. Pathway mapping demonstrated inflammation mediated by chemokine, cytokine, and angiogenesis as one of the top pathways with a significantly higher number of transcripts in db/db mice. These molecular changes were reflected in an overt defect in LSK mobility in the bone marrow. miRNA downstream target analysis unveils several mRNAs targeting leukocyte migration, microglia activation, phagosome formation, and macrophage activation signaling as their primary pathways, suggesting a shift to an inflammatory phenotype.

Conclusion Our findings highlight that chronic diabetes adversely alters HSCs' homeostasis at the transcriptional level, thus potentially contributing to the inflammatory phenotype of HSCs under long-term diabetes. We also believe that identifying HSCs-based biomarkers in miRNAs or mRNAs could serve as diagnostic markers and potential therapeutic targets for diabetes and associated vascular complications.

Keywords Hematopoietic stem cells, Chronic diabetes, Transcriptome, Inflammation

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Background

Diabetes is a chronic debilitating metabolic disease affecting more than 537 million people worldwide, and this number is predicted to rise to 783.2 million by 2045 [1, 2]. Hematopoietic stem cells (HSCs) are bone-marrow-derived stem cells that can differentiate into myeloid, lymphoid cells, platelets, and red blood cells [3]. These bone marrow-derived HSCs are adversely affected by chronic diabetes. With the primary role of tissue maintenance, homeostasis, and vascular repair, these HSCs in diabetes lose their ability to be reparative, instead, it has been reported in multiple studies that mobilopathy, stem cell rarefaction, neuropathy, micro-angiopathy, and inflammation become more prevalent in HSCs with diabetes [4, 5]. Diabetes also manifests many micro- and macro-vascular complications, such as retinopathy, neuropathy, cardiomyopathy, and nephropathy, posing a high risk for multi-organ damage [6]. Consistently, it has been shown that low levels of HSCs in chronic diabetes accelerate the progression of diabetes-induced microvascular complications [7, 8].

The transcriptomics studies have strengthened our knowledge of genetic factors associated with the development and associated outcomes of diabetes. Importantly, different groups have utilized the insulin-targeted organs, such as the pancreas, liver, skeletal muscles, adipose tissues, etc., for gene expression studies reflecting the dominance of inflammatory immune responses in the pathogenesis of diabetes [9–11]; however, Lin⁻Sca1⁺c-Kit⁺ cells' (LSK) transcriptome under long-standing diabetes has not been studied. In addition to mRNA targets, non-coding RNAs like micro RNAs (miRNA) have been widely explored for their role in chronic diabetes progression [12]. Recent evidence has shown the potential of tissue-specific and circulating miRNAs for their capability to be used as a biomarker in diabetes and associated vascular complications [13, 14]. Therefore, studying miRNA and mRNA transcriptomic could provide critical information about LSK cell phenotype under conditions of long-standing diabetes.

It is noteworthy that HSC dysfunction is a known outcome of diabetes [4, 5]; however, the transcriptome signature of these HSCs in diabetes has not been investigated. In the present study, we isolated a highly purified fraction of HSCs; Lin⁻Sca1⁺c-Kit⁺ (LSK) cells from the bone marrow of db/db mice (an animal model of type 2 diabetes) with six months of diabetes followed by miRNA and mRNA sequencing. Our studies unravel previously unknown miRNAs and mRNA transcripts changed with long-term diabetes, which could have contributed to HSC mobilopathy and inflammatory phenotype.

Methods

Animals

The B6.BKS(D)-Lepr^{db}/J (an animal model for T2D; db/db; $n=9$) and Lepr^{db/+} db/m (heterozygotes; db/m; $n=9$) mice [stock number 000697] were procured from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in the animal care facility at Glick Eye Institute, Indiana university. All the animals were kept under normal physiological conditions (12-hour light/dark conditions), with access to food and water *ad libitum*. The mice were maintained for eight months (6 months of diabetes) before proceeding with the bone marrow experiments. All the experiments were performed per the Guiding Principles in the Care and Use of Animals (National Institutes of Health). The mice were euthanized using inhalation overdose of CO₂ delivered using a gas cylinder, /regulator, and induction chamber.

Hematopoietic stem cell (HSCs) isolation

Femur and tibia were collected for bone marrow isolation after animal sacrifice. Briefly, all the surrounding muscles were removed, and bones were cleaned and flushed with FACS buffer (PBS with 2% FBS) with the help of a needle. A cell suspension was generated by triturating the cells through the needles. Cells were then treated with ice-cold ammonium chloride for 10 min to lyse all the red blood cells, followed by washing with PBS and staining with an anti-mouse lineage cocktail, Sca1, and c-Kit/CD117 antibodies (STEMCELL Technologies) for 30 min. The samples were finally sorted for lin⁻Sca1⁻ckit⁺ cells using a flow cytometer. The pure population of lin⁻Sca1⁻ckit⁺ cells represents HSCs used for the sequencing experiments.

RNA isolation and sequencing

The RNA was extracted from the sorted HSCs using the Trizol-chloroform method followed by DNase treatment in the solution and cleaned up with an RNeasy MinElute clean-up kit from Qiagen. The purity of RNA was confirmed on the Bioanalyzer before proceeding with the sequencing. Only the samples with a RIN (RNA integrity number) of >7 were used for the sequencing ($n=4$ per group). The samples were prepared at the medical genomics core facility (Indiana University) for the paired-end mRNA and single-end miRNA seq library and sequenced on Illumina NovaSeq 6000 with a read length of 100 bp for mRNA and NextSeq 2000 with a read length of 75 bp for miRNA respectively. The mRNA sequence reads were mapped to the designated reference genome (UCSC version mm10; also used as a genome annotation file) using STAR version 2.7.10a (Spliced Transcripts Alignment to a Reference) [15]. Adaptor trimming was not required with the aligner STAR. To evaluate the quality of the RNA-seq data, the number of

reads that fall into different annotated regions (exonic, intronic, splicing junction, intergenic, promoter, UTR, etc.) of the reference genes was determined with bamUtils [16]. Low-quality mapped reads (including reads mapped to multiple positions) were excluded, and featureCounts [17] were used to quantify the expression of gene level. The differential gene expression analysis for mRNA was performed with edgeR version 3.42.0 [17]. In this workflow, the statistical methodology uses negative binomial generalized linear models with likelihood ratio tests. The miRNAs raw sequencing reads were subjected to FASTQC for quality control (Table S1). No adaptor trimming was required. The miRNA sequence reads were mapped to the designated reference genome (miR-Base_v22 version *Mus musculus* (GRCm38.101)) using the Qiagen GeneGlobe RNA-seq Analysis Portal version 4.0 with default parameters for miRNA and the differential gene expression analysis for miRNA was performed with edgeR version 3.41.9 [17]. The Benjamini-Hochberg (BH) procedure was used to correct the multiple statistical tests to get the adjusted *p*-values.

Pathway analysis

Ingenuity pathway analysis (IPA) software (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) was used to identify biological/molecular pathways associated with the differentially expressed miRNAs and their targets. Both miRNAs and mRNA data were uploaded to the IPA and were subjected to the threshold filter of *p*-value -0.05 . Then, a Micro RNA target filter was applied to find the mRNA targets for the differentially expressed miRNAs. The filter selections for our analysis included $p < 0.05$, experimentally observed interactions, and direct and indirect relationships. mRNA targets from the IPA were overlaid with the mRNA targets from our sequencing database, and finally, pathways analysis was done for those miRNAs and mRNAs. Significant mRNAs ($p < 0.05$) were also subjected to Gene Ontology and Panther analysis (pantherdb.org).

qRT-PCR for miRNAs and mRNA targets

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. 10 ng of total RNA and 1 μ g of RNA were reverse transcribed using TaqMan miRNA reverse transcription kit (4366596; Thermo Fisher Scientific) and cDNA Synthesis Kit (SuperScript VILO; Thermo Fisher Scientific) for miRNA and mRNA, respectively. Gene-specific primers were used along with a master mix (TaqMan Fast Universal; Thermo Fisher Scientific) and respective miRNA and mRNA levels were determined using quantitative PCR (Vii7; Thermo Fisher Scientific). All miRNAs were normalized to U6 and the mRNA genes were normalized

to TBP, and data was analyzed using $\Delta\Delta$ Ct method [18]. Primers used were *Gpr89* (Mm00509312_m1), *Pla2g4c* (Mm01195718_m1), *Cxcl12* (Mm00495553_m1), *Il10* (Mm01288386_m1), *Csf1* (Mm00432686_m1), *Itga5* (Mm00439797_m1), *Tbp* (Mm00446973), mmu-miR-3968 (463749_mat), mmu-miR-1971 (121161_mat), mmu-miR-148a-3p (000470), mmu-miR-126a-5p (4427975) and miRNA U6 (001973).

Statistical analysis

All the data were expressed as Mean + SEM and analyzed using GraphPad Prism 10.0.1 for Windows (San Diego, California; www.graphpad.com). The intergroup comparison was done using a t-test and was considered significant when the *p*-value was less than 0.05.

Results

The bone-marrow-derived HSCs and diabetes

Long-term diabetes adversely affects the bone marrow's physiological function, including the stem cell niche, [4], however, the transcriptional signature of HSCs under diabetes remains unelucidated. Therefore, we evaluated the pure population of HSCs using miRNA and mRNA sequencing (Fig. 1a). The bone marrow was first subjected to flow cytometric sorting to isolate the LSK cells. Assessment of the pure LSK cell population showed an increase in db/db mice (Fig. 1b), indicating bone marrow mobilopathy with chronic diabetes. The pure population of LSK cells was then used for the transcriptome analysis. Out of 13,708 genes with non-zero total read count; 2076 genes were significantly expressed in HSCs of db/db mice ($p < 0.05$) (Fig S1a). The volcano plot represents the total genes identified (Fig S1b), whereas the heatmap in Fig. 1c represents significantly changed genes between db/m and db/db mice. Of these significantly changed genes, IPA analysis revealed that 1036 were upregulated, whereas 944 were downregulated in the db/db mice (Fig S1a).

miRNA sequencing found a total of 191 miRNA, out of which 35 miRNAs were significantly changed in db/db mice ($p < 0.05$) (Fig. 1d). Figure 1e represents the volcano plot for a total of 191 miRNAs observed from the sequencing, whereas the top 35 miRNAs were presented in a heatmap in Fig. 1f. The differentially expressed miRNAs [35] were then subjected to Ingenuity pathway analysis (IPA) for a deeper understanding of their involvement in various molecular pathways. The core analysis from IPA showed the top 10 upregulated and downregulated miRNAs in db/db mice (Table 1). Micro RNA target filter analysis *via* IPA software showed that out of 35 significantly changed miRNAs, 28 miRNAs had 1974 mRNA targets. Further, these mRNA targets were traced back to the mRNAs identified in the LSK cells of the present study, which revealed that 446 mRNAs were

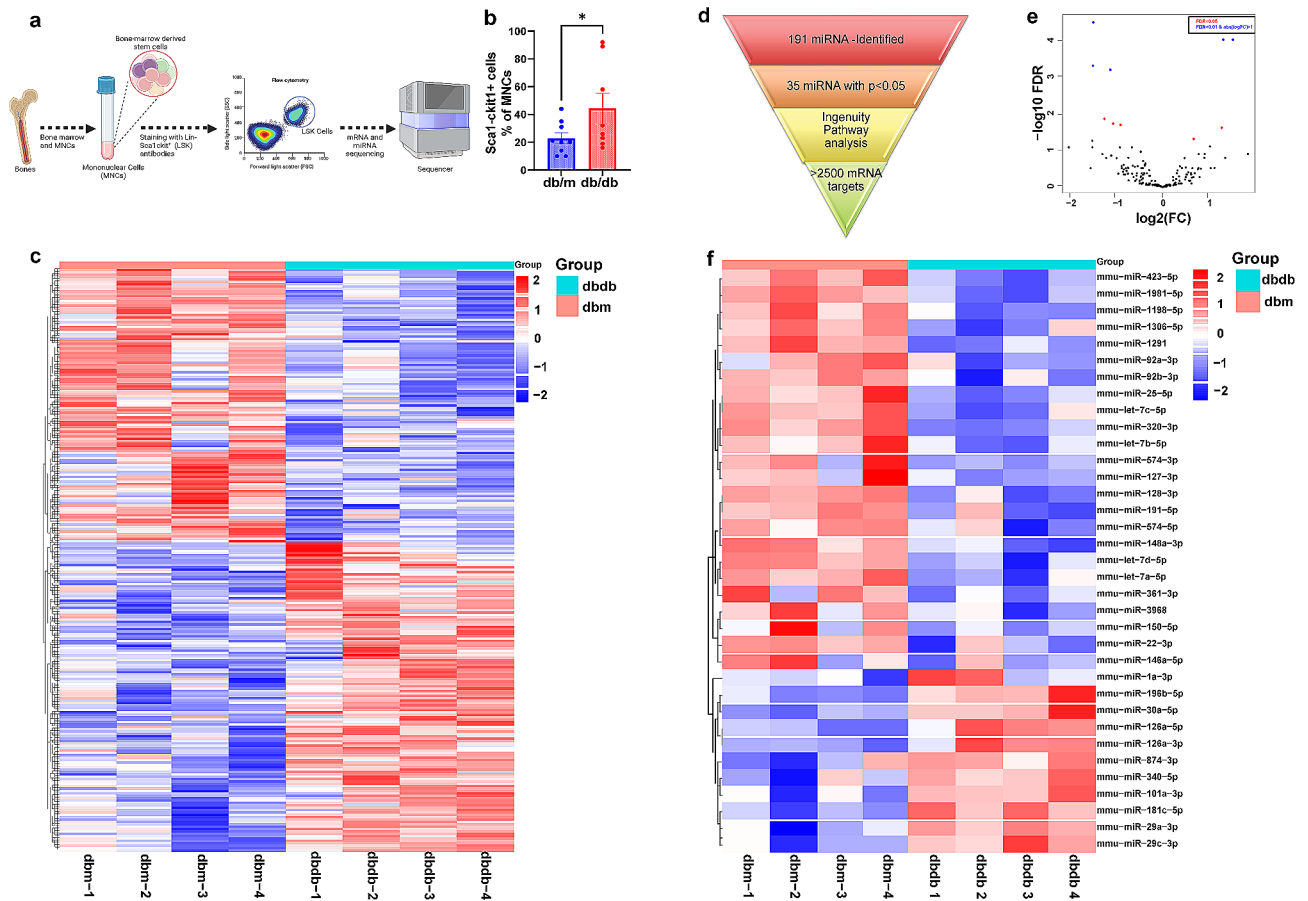


Fig. 1 Transcriptome analysis of HSCs in diabetes. **(a)** Schematic presentation of the steps followed for isolating, sorting, and sequencing HSCs from the bone marrow. **(b)** The sorted HSCs population was found to be higher in the db/db mice. **(c)** Heatmap depicting the significantly changed mRNAs between db/m and db/db mice. **(d)** The representative procedure for the miRNA data analysis. **(e)** The volcano plot for the total miRNAs found in the sequencing data. The red and blue dots represent the significantly changed miRNAs. **(f)** The top 35 miRNAs are presented as a heatmap. The data in Figure **(b)** is presented as mean ± SEM, analyzed using student unpaired t-test; N=9. The heatmaps in Figures **(c)** and **(f)** were plotted using a freely available online tool SRplot (<https://www.bioinformatics.com.cn/en>)

also present in our mRNA sequencing data; on the other hand, 1528 genes or mRNA targets were unique or were absent in our mRNA sequencing data (Fig S1c). These miRNAs had several downstream targets, some unique to the individual miRNA, and some overlapping between multiple miRNAs (Fig S1d).

Transcriptome analysis of bone-marrow-derived LSK cells in chronic diabetes

Gene ontology using the online available software Panther was performed to investigate the molecular pathways associated with the differential mRNAs in the LSK cells. It was found that most of the genes were associated with inflammation mediated by chemokine and cytokine signalling pathways (Fig. 2a). Interestingly, angiogenesis was also one of the top pathways, with a significantly higher number of genes in the transcriptome of db/db mice’s LSK cells (Fig. 2a). A significant increase in the angiogenic genes, such as *Vegfc*, *Pdgfd*, *Angpt1*, *Tgfb1i1*,

Pla2g4c, and *Hif3a*(Fig. 2b) was found in the diabetic LSK cells. We also found an increase in the expression of *Cxcl12*, *Cxcl5*, and *Csf1*(Fig. 2c). No significant change was observed in the expression level of *Cxcr4* in LSK cells (Fig. 2c). To study inflammatory changes, we investigated our mRNA data for major inflammation-related genes, including Toll-like receptors (TLRs), interleukins (ILs), and tumour necrosis factor (TNF). The anti-inflammatory cytokine *Il10* was significantly downregulated in the HSCs of db/db mice (Fig. 2d). In contrast, a significantly higher expression of *Il4*, *Tlr4*, and *Tnf11a* (Fig. 2e) was found in the HSCs of db/db mice.

Overexpression of inflammatory pathways in the HSCs of db/db mice

For further pathway analysis, we chose 446 genes that were miRNA targets and were also present in our mRNA sequencing database. Interestingly, out of 446 genes, 90 were targeted by multiple miRNAs (Fig. 3). These target

Table 1 Top analysis ready MicroRNAs (10 upregulated and 10 downregulated)

Sr No.	MiRNAs	Normalized Expression: Log Fold Change/ LogFC	Change of Expression
1	miR-1-3p (and other miRNAs w/seed GAA AUGU)	1.841	Upregulated
2	miR-126a-5p (and other miRNAs w/seed AUUAUUA)	1.520	Upregulated
3	miR-874-3p (miRNAs w/seed UGCCUG)	1.351	Upregulated
4	miR-126a-3p (and other miRNAs w/seed CGUACCG)	1.311	Upregulated
5	miR-30c-5p (and other miRNAs w/seed GUAACA)	1.277	Upregulated
6	miR-101-3p (and other miRNAs w/seed ACAGUAC)	1.022	Upregulated
7	miR-181a-5p (and other miRNAs w/seed ACAUUCA)	0.911	Upregulated
8	miR-340-5p (miRNAs w/seed UAUAAAG)	0.904	Upregulated
9	miR-29b-3p (and other miRNAs w/seed AGCACCA)	0.783	Upregulated
10	miR-196a-5p (and other miRNAs w/seed AGGUAGU)	0.672	Upregulated
11	miR-3968 (miRNAs w/seed GAAUCCC)	2.011	Downregulated
12	miR-22-3p (miRNAs w/seed AGCUGCC)	1.510	Downregulated
13	miR-574-5p (miRNAs w/seed GAGUGUG)	1.503	Downregulated
14	miR-148a-3p (and other miRNAs w/seed CAGUGCA)	1.495	Downregulated
15	miR-423-5p (and other miRNAs w/seed GAGGGGC)	1.487	Downregulated
16	miR-127-3p (miRNAs w/seed CGGAUCC)	1.291	Downregulated
17	miR-1971 (and other miRNAs w/seed UAAAGGC)	1.245	Downregulated
18	miR-361-3p (miRNAs w/seed CCCCAG)	1.209	Downregulated
19	miR-320b (and other miRNAs w/seed AAAGCUG)	1.120	Downregulated
20	miR-1306-5p (miRNAs w/seed ACCACCU)	1.100	Downregulated

mRNAs could be of more potential as their regulation is controlled by more than one miRNA. We ran a core pathway analysis on significantly changed miRNAs to investigate the potential pathways associated with differentially expressed miRNAs. The top 5 miRNAs (both upregulated and downregulated) (Table 1) were further subjected to the network analysis and overlaid with the mRNA from our sequencing database. Since our group has previously shown the role of miR-92 in diabetic retinopathy [19, 20], we also included miR-92 (fold change -1.577 and $p=0.0065$) for the network analysis. Lastly, these top miRNAs were subjected to the IPA pathway analysis.

IPA pathway and network analysis showed that each miRNA was targeting inflammation-related pathways. miRNAs are a known transcriptional regulator, which can bind to the mRNA's 3'-UTR regions, leading to their degradation. With the differential expression of the top regulated miRNAs [miR-874-3p, miR-30c-5p, miR-3968 and miR-148a-3p (Fig. 4a-d); and miR-1-3p, miR-423-5p, miR-22-3p, miR-574-5p, and miR-92a-3p (Fig S2-S4) of the LSK cells, we found an increase in the expression of downstream targets like colony-stimulating factor 1 (CSF1), Kruppel-like factors (*Klf6*, *Klf4*, and *Klf2*), integrins (*Itga5*, *Itga9*, *Itgav*, *Itgb3*), and collagen (*Col1a1*, *Col1a2*, *Col5a1*, *Col4a2*) all of which held the potential to stimulate the chemotaxis, differentiation and proliferation of bone-marrow-derived monocytes/macrophages and microglia. The top canonical pathways affected by these miRNAs and their respective targets turned out to be macrophage alternative activation signalling,

neuroinflammation, IL-4 signalling, TGF- β signalling, CCR5 signalling in macrophages, leukocyte extravasation signalling, VEGF family ligand-receptor interactions, neurovascular coupling signalling, neutrophil extracellular trap signalling, phagosome formation, WNT/Ca⁺ signalling, IL-12 signalling, natural killer cell signalling and CREB signalling in neurons (Fig. 4a and Fig S2, 3, 4). The IPA suggested a shift to the pro-inflammatory phenotype of HSCs in the db/db mice at the transcriptional level, which in turn is largely regulated by the miRNAs.

RT-PCR validation of top miRNAs and their targets

Finally, we validated two of the novel miRNAs observed in the sequencing data. Consistent with the sequencing data, we found the downregulation of miR-3968 and miR-1971 in db/db mice (Fig. 5a). Next, we also checked the qPCR expression of miR-148a-3p (top-downregulated miRNA), and miR-126a-5p (reported to be downregulated in chronic diabetes). As observed in our sequencing data, miR-126a-5p was upregulated, and miR-148a-3p was downregulated in LSK cells of db/db mice (Fig. 5a). Since these miRNAs were significantly affected, it was interesting to investigate the expression patterns of their mRNA targets. The top mRNA targets, such as *Pla2g4c* (miR-3968 target) and *Gpr89a/b* (miR-1971 target), significantly changed in gene expression analysis (Fig. 5b). *Csf1* and *Itga5* (miR-148a-3p target) were found to be upregulated; however, this difference was non-significant for *Csf1* (Fig. 5b). Further, the gene expression of *Il-10* (an anti-inflammatory gene from Fig. 2b) was significantly downregulated and *Cxcl12* (participates in HSCs

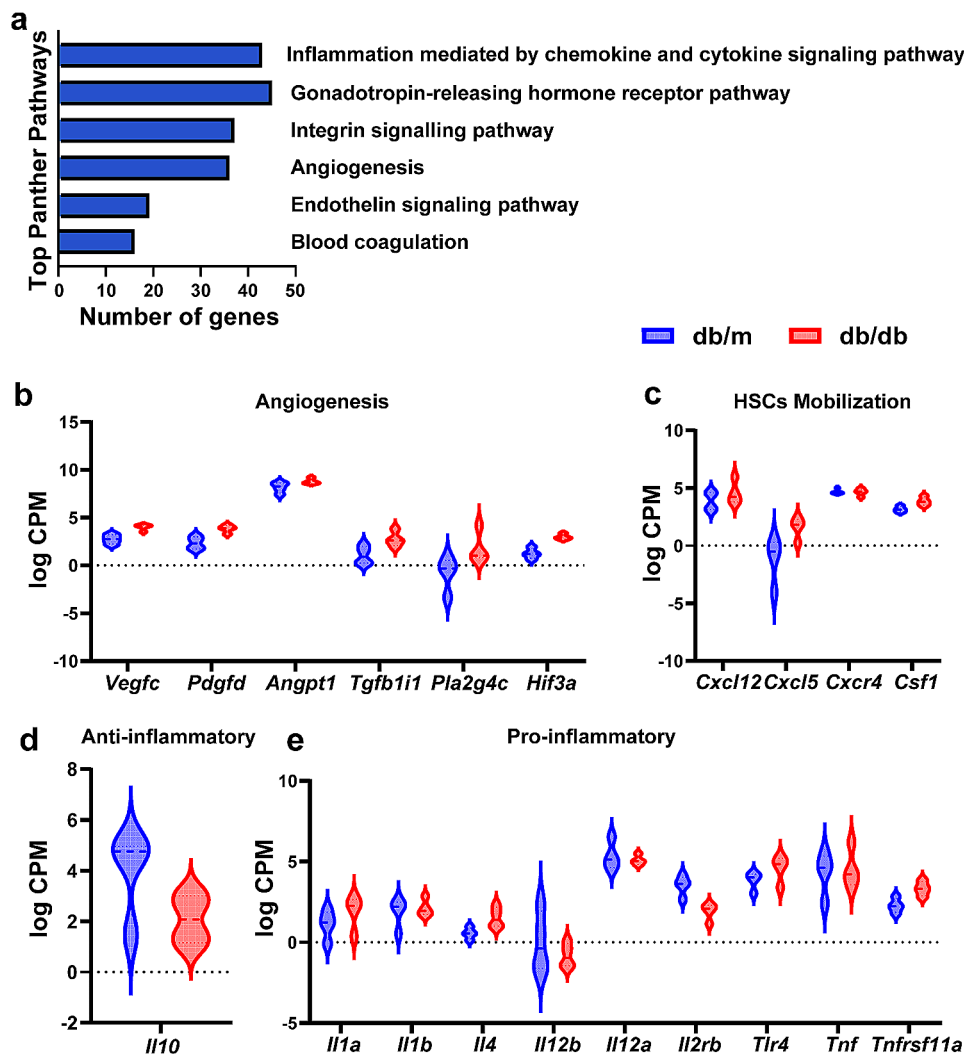


Fig. 2 Transcriptome analysis revealed the gene level changes in the HSCs of db/db mice. The significant changes in mRNAs were analyzed using Gene ontology/Panther to uncover the top pathways. **(a)** The pathways with the highest number of differentially expressed genes in the HSCs of db/db and db/m mice. Individual pathways analysis showed increased expression of **(b)** angiogenesis, **(c)** differentiation, and migration of HSCs-related genes. **(d)** The anti-inflammatory gene IL-10 was significantly downregulated in the db/db mice, **(e)** whereas there was an increase in the expression level of pro-inflammatory genes in db/db mice

mobilization) was also found to be significantly upregulated in db/db mice (Fig. 5b)

Discussion

In the present study, we have investigated the transcriptional signature of bone marrow-derived HSCs under chronic diabetes, which has not been explored before. These transcriptional changes can, therefore, change the fate of circulatory HSCs. Through our transcriptional profiling, we identified two novel miRNAs (miR-3968 and miR-1971) and a distinguished set of downstream mRNAs targeting pro-inflammatory, leukocyte migration, and angiogenesis-related pathways in HSCs of diabetic animals with the potential to participate in diabetes-induced vascular complications.

Bone marrow-derived HSCs are widely studied and used as therapeutics for malignant blood disorders due to their outstanding capacity to regenerate the hematolymphoid system [21]. In the last two decades, multiple studies have reflected the negative outcome of diabetes on the HSCs, in terms of bone marrow function, release and migration ability of HSCs, and decreased circulation in the peripheral blood [22]. It is well-appreciated now that chronic diabetes can lead to an impaired endothelial repair system and promote stem cell mobilopathy and neuropathy [5, 23–25]. Consistent with the previous literature, db/db animals in the present study had a significantly higher number of entrapped HSCs in the bone marrow [25]. Stem cell mobilization is largely dependent on the CXCL12-CXCR4 signalling, where the CXCL12 upregulation is linked to impaired stem cell trafficking/

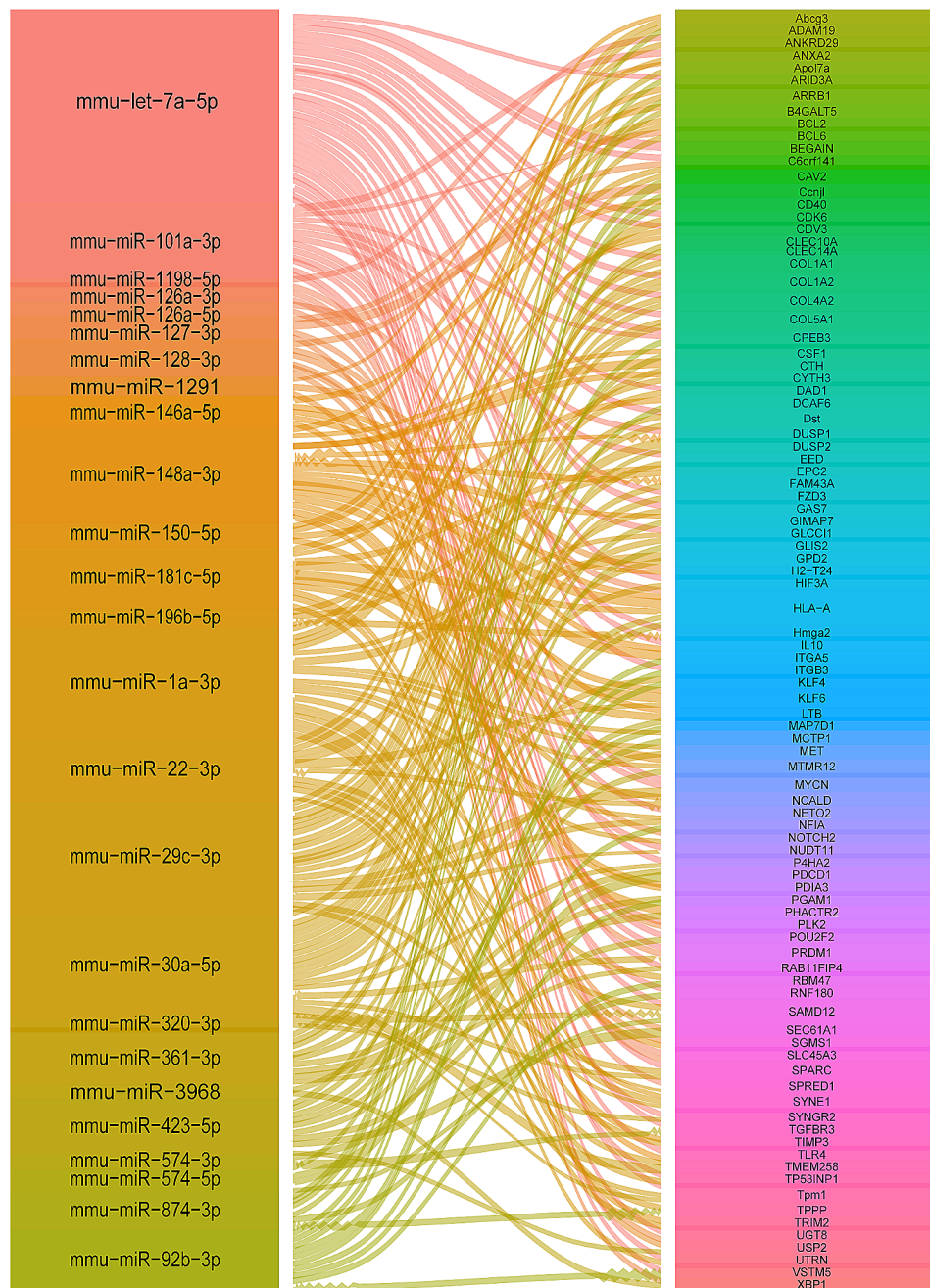


Fig. 3 Alluvial plot illustrating overlapped mRNA targets between multiple miRNAs. MicroRNA target filter analysis revealed a total of 1974 mRNA targets, which were then overlaid with the significantly expressed mRNA targets. 446 mRNA targets were common among two datasets, of which 206 mRNAs were targeted by multiple miRNAs, as shown in the Figure. This Figure was plotted using a freely available online tool SRplot (<https://www.bioinformatics.com.cn/en>)

mobilopathy in diabetes [26, 27]. The higher expression of *Cxcl12*, with the simultaneous increase in the entrapped HSCs, reflected the inability of the HSCs to be released peripherally in diabetic animals.

The present study is the first of this kind, where the pure LSK cells were sorted and utilized to investigate diabetes-induced transcriptional changes in the db/db mice. Further, there is growing evidence that miRNAs

play a crucial role in the transcriptional regulation of enormous molecular targets associated with metabolic disorders, including diabetes or diabetes-induced vascular complications [28–33]. However, it is noteworthy that most of the above studies have been done on circulatory or tissue-specific miRNAs, and no data is available for the HSCs miRNAs. Therefore, we investigated the LSK miRNA and mRNA targets due to their

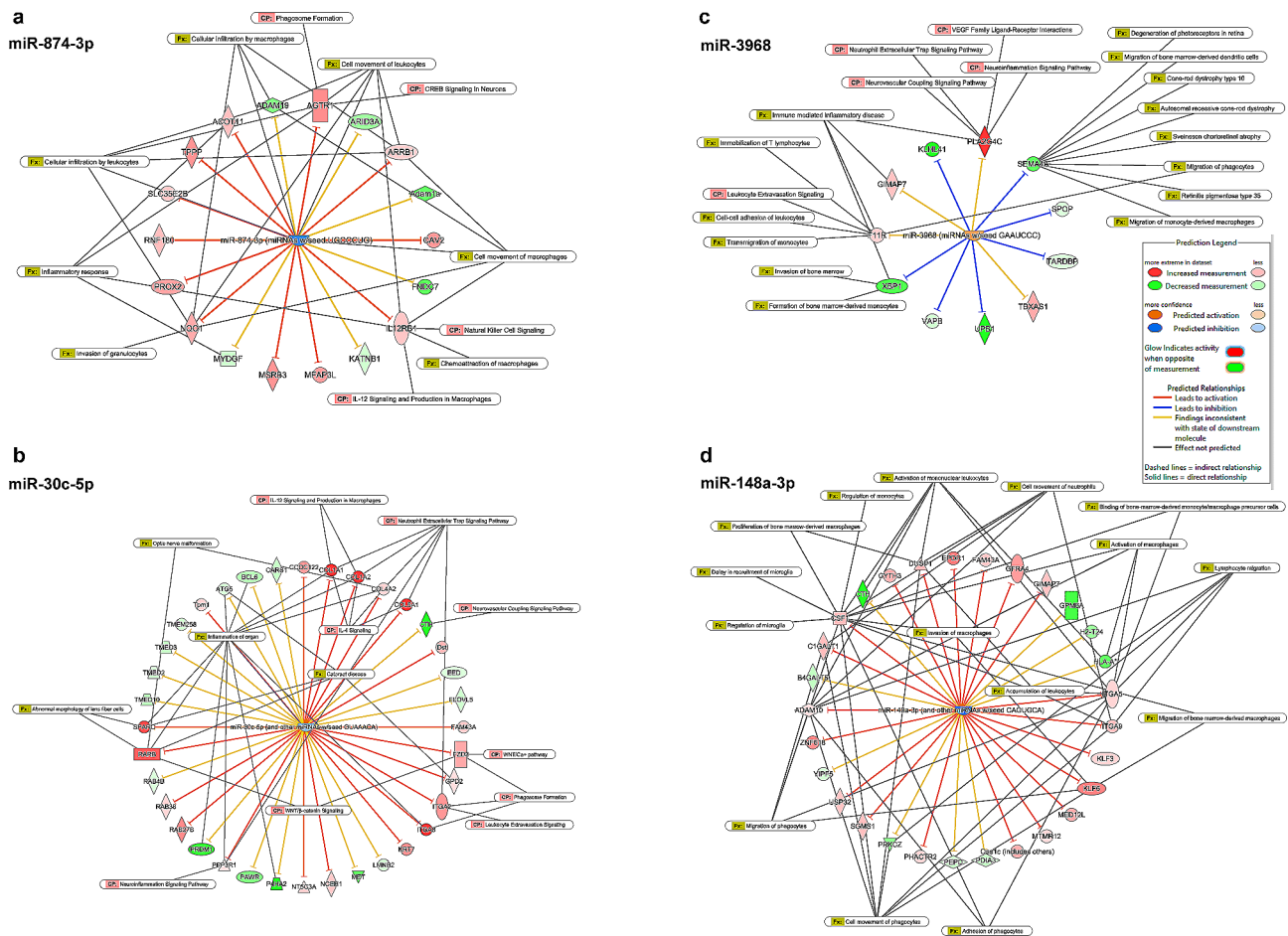


Fig. 4 Top pathway analysis for miRNAs reflected increased inflammation-related genes in db/db mice. Top miRNAs were analyzed using IPA software for their downstream targets and associated pathways. **(a)** miR-874-3p and **(b)** miR-30c-5p were upregulated in the HSCs of db/db mice, which were traced back to leukocyte extravasation, neuroinflammation, cytokine signaling, and macrophage signaling. **(c)** and **(d)** miR-3968 and miR-148a-3p were top downregulated miRNAs, targeting VEGF signaling, formation, proliferation, and migration of bone marrow-derived macrophages and monocytes, recruitment, and migration of microglia, neuroinflammation and degeneration of photoreceptors. The data was uploaded to the IPA, and miRNAs and mRNAs with a $p < 0.05$ were used for the miRNA target filter and core pathway analysis

ability to change the fate of the stem cells being circulated and used for vascular repair. We found 35 significantly changed miRNAs in the LSK cells of db/db mice, with 25 being downregulated and 10 being upregulated. Interestingly, our sequencing data has uncovered 2 novel miRNAs (miR-3968, and miR-1971), which have never been reported before for any involvement with diabetes or diabetes-induced microvascular complications; previously, miR-3968 was downregulated in gestational diabetes [34] and our study presented the similar change in the LSK cells of diabetic mice. Additionally, our RT-PCR validation supported the expression pattern of these two novel miRNAs being downregulated in chronic diabetes. There were several miRNAs consistent with the reported literature, including miR-1-3p [35, 36], miR-30c-5p [37–39], miR-101-3p [40, 41], miR-181a-5p [42, 43], miR-340-5p [44], miR-29b-3p [43, 45, 46], miR-423-5p [47] and miR-22-3p [12, 48, 49]. Contrarily, we found three

miRNAs (miR-126a-5p and miR-126a-3p [33, 50]; and miR-148a-3p [50] inconsistent with the reported miRNAs in terms of their expression pattern. Intriguingly, our quantitative real-time PCR analysis also reflected the downregulation of miR-148a-3p and upregulation of miR-126a-5p, with no change in miR-126a-3p expression. Since this study is distinguishable from the available literature due to the use of HSCs, the miRNAs observed in the data hold great potential irrespective of their expression difference with the previous studies.

The miRNAs target several downstream pathways that can manifest the fate of the stem cells, to explore this phenomenon, we further analyzed the top miRNAs. We found multiple miRNAs and their downstream mRNAs targeting inflammatory and angiogenic pathways. One of the top targets of miR-3968, which is *Pla2g4c*, was significantly upregulated under chronic diabetes. Phospholipase A2 (PLA2) is a family of lipolytic enzymes that

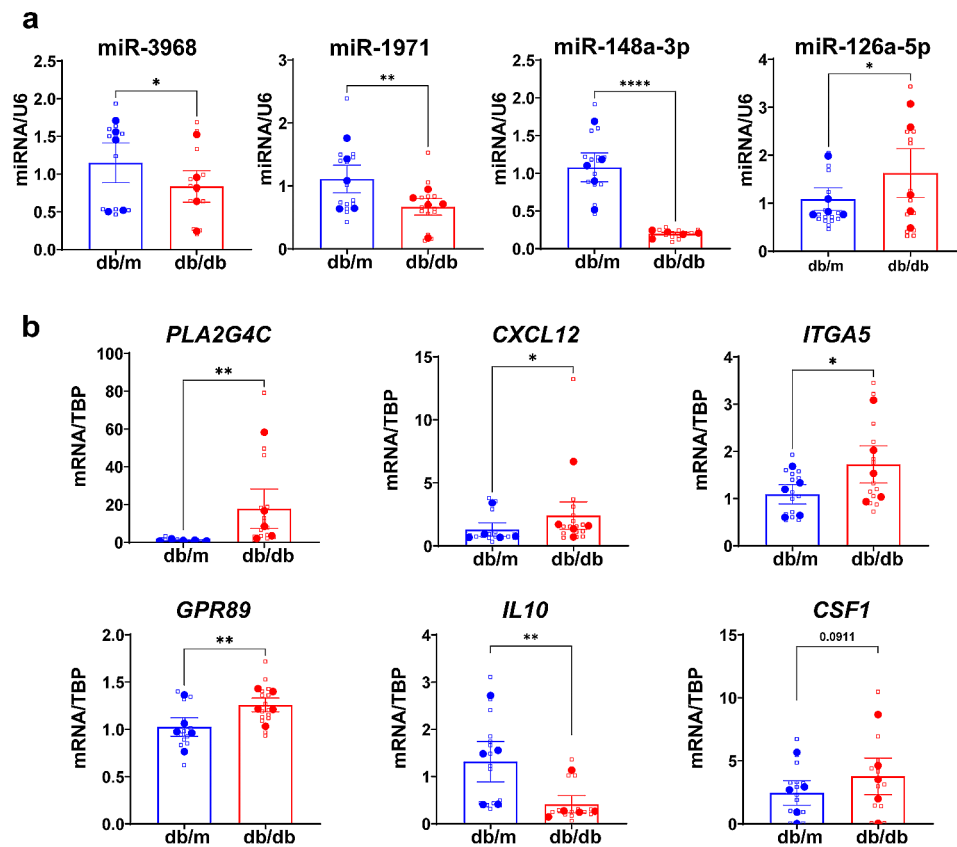


Fig. 5 Validation of novel miRNAs and mRNA using qPCR in db/db mice. The top 3 downregulated miRNAs (miR-3968, miR-1971 & miR-148a-3p), miR-126a-5p, and their mRNA targets were analyzed using real-time quantitative PCR (qRT-PCR). **(a)** Gene expression analysis of miR-3968, miR-1971, miR-148a-3p, and miR-126a-5p normalized to U6 **(b)** Gene expression analysis of mRNA targets normalized to TBP. The figure is presented as a SuperPlot generated using GraphPad Prism, where solid dots represent biological replicates (mean \pm SEM; $N=5$) and small square boxes represent technical replicates. The data are analyzed on technical replicates using student unpaired t-test ($N=5*3=15$)

can produce lipid pro-inflammatory substances such as free fatty acids (FFA) that participate in inflammatory response. PLA2 family activation has been reported in diabetic retinopathy [51], diabetic nephropathy [52, 53], and cardiovascular complications. A significant upregulation of *Pla2g4c* in LSK cells of diabetic animals suggested the involvement of lipid-induced inflammatory changes, which could be explored further. Notably, platelet-derived growth factor (PDGF) also activates phospholipases, releasing prostaglandins [53]. Our present transcriptome analysis reflected these observations, where a simultaneous upregulation of *Pdgf* and *Pla2g4c* was found, potentially corresponding to the gonadotrophin-releasing hormone receptor pathways. The downstream target of miR-1971 was *GPR89a/b*, which has not been studied so far for its direct role in diabetes or diabetes-induced vascular complications. Herein, we could not validate the downregulation of *Gpr89a/b* with RT-PCR.

Another target is Kruppel-like factors (KLFs); a family of transcription factors that regulate macrophage differentiation and migration under the influence of inflammatory signals [54]. Interestingly, there was a significant

increase in the expression levels of *Klf4* and *Klf6* in the LSK cells of diabetic animals, possibly contributing to the migration of inflammatory monocytes/macrophages [55–57]. Moreover, we also found a decrease in the expression of miR-92a-3p in the LSK cells, which was previously shown to be decreased in the angiogenic cells of diabetic retinopathy individuals [58] and retinas of diabetic mice [59]. Also, it has been shown that interactions of miR-92a-3p with KLF2 and KLF4 can modulate inflammatory macrophage signaling [60, 61], thus highlighting the possible involvement of miR-92a-3p regulated inflammatory pathways in diabetic HSCs. Likewise, studies have shown the role of colony-stimulating factor 1 (CSF1) in microglia activation and inflammatory cytokine secretion [62]. We also found a higher expression of the *Csf1* gene in diabetic animals, thus promoting microglia activation and inflammation. A higher expression of pro-inflammatory genes *Tlr4*, *Il1 α* , and *Tnf11 α* and a decrease in the expression levels of anti-inflammatory cytokine *Il10*, in the LSK cells, also suggested a shift to inflammatory phenotype [63, 64] in diabetic HSCs. Moreover, the *Ccl4* gene, which regulates macrophage

activation, chemotaxis, and migration, was expressed more strongly. A simultaneous increase of these genes in the LSK cells of db/db mice marks the initiation of inflammation at transcriptional levels in the stem cells.

In addition to the changes in the inflammatory genes, we found several angiogenic genes being upregulated in the LSK cells of db/db mice. Vascular endothelial growth factor (VEGF) is a widely studied angiogenic factor with multiple isoforms [65]. VEGF-C, primarily known for lymphangiogenesis, has also been reported to play a part in pathological angiogenesis [66]. An increase in the gene expression of *Vegfc* in db/db mice reflects the angiogenic fate of HSCs.

Conclusion

In conclusion, we have observed HSCs mobilopathy and adverse effects on the transcriptome profile of LSK cells in chronically diabetic animals. Several miRNAs and mRNA targets reflected a shift to the inflammatory and angiogenic fate of bone-marrow-derived HSCs in diabetes. The investigation of HSCs transcriptome is clinically very relevant as these cells not only participate in the pathogenesis of disease but also play an important role in hematopoiesis and vascular repair. We believe that the present study has strengthened the current knowledge of diabetes-induced HSC dysfunction at the transcriptional levels, with the potential to contribute to vascular damage. Identifying novel miRNAs and their targets could be a potential biomarker for diabetes-induced vasculature deficits such as retinopathy. However, further studies are required to investigate the molecular mechanisms linking the HSCs miRNAs or mRNA targets to the progression of vascular disease in diabetes.

Abbreviations

LSK	Lin ⁻ Sca1 ⁺ c-Kit ⁺ cells
HSCs	Hematopoietic Stem Cells
miRNA	Micro RNA
T2D	Type 2 Diabetes
IPA	Ingenuity pathway analysis
TLRs	Toll-like receptors
ILs	Interleukins
TNF	Tumour necrosis factor
KLFs	Kruppel-like factors
CSF1	Colony-stimulating factor 1
VEGF	Vascular endothelial growth factor

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-024-10679-3>.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5

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Author contributions

Conceptualization: AB, NM; Validation: NM, AB; Formal Analysis: NM, AB; Investigation: NM, QL; Resources: AB; Data Curation: NM, QL, SA; Writing-Original Draft: NM; Writing-Review & Editing: AB, NM; Visualization: NM, AB; Supervision: QL, AB; Project Administration: AB, QL, NM; Funding Acquisition: AB.

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Data availability

Transcriptional data generated in this study was deposited in National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession no. GSE261501 (mRNA data) and GSE261502 (miRNA data).

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Indiana University [Approval no. 210611]. We declare that all the experiments performed were per the Guiding Principles in the Care and Use of Animals (National Institutes of Health) and in compliance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

AB is an ad hoc District Support Pharmacist at CVS Health/Aetna. The contents of this study do not reflect those of CVS Health/Aetna. NM, QL, and SA do not have any conflicts to declare.

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