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Circulating miRs-183-5p, -206-3p and -381-3p may serve as novel biomarkers for 4,4'-methylene diphenyl diisocyanate exposure

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Abstract

Background: Occupational exposure to the most widely used diisocyanate, 4,4'-methylene diphenyl diisocyanate (MDI), is a cause of occupational asthma (OA). Early recognition of MDI exposure and sensitization is essential for the prevention of MDI-OA.

Objective: Identify circulating microRNAs (miRs) as novel biomarkers for early detection of MDI exposure and prevention of MDI-OA.

Materials and methods: Female BALB/c mice were exposed to one of three exposure regimens: dermal exposure to 1% MDI in acetone; nose-only exposure to 4580 ± 1497 μg/m^3 MDI-aerosol for 60 minutes; or MDI dermal exposure/sensitization followed by MDI-aerosol inhalation challenge. Blood was collected and miRCURY™ miRs qPCR Profiling Service was used to profile circulating miRs from dermally exposed mice. Candidate miRs were identified and verified from mice exposed to three MDI-exposure regimens by TaqMan® miR assays.

Results: Up/down-regulation patterns of circulating mmu-miRs-183-5p, -206-3p and -381-3p were identified and verified. Circulating mmu-miR-183-5p was upregulated whereas mmu-miRs-206-3p and -381-3p were downregulated in mice exposed via all three MDI exposure regimens.

Discussion and conclusion: Upregulation of circulating miR-183-5p along with downregulation of circulating miRs-206-3p and -381-3p may serve as putative biomarkers of MDI exposure and may be considered as potential candidates for validation in exposed human worker populations.

Keywords

Occupational Asthma (OA); Diisocyanates (dNCOs); 4,4'-methylene diphenyl diisocyanate (MDI); Circulating microRNAs (miRs)

Introduction

Diisocyanates (dNCOs) are low molecular weight cross-linkers used in polyurethane production. Methylene diphenyl diisocyanate (MDI) is the most widely used dNCO globally

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(Allport *et al.*, 2003), where it is utilized in spray foam insulation, truck bed liners, wood products and adhesives. MDI is a potent respiratory system and skin irritant/sensitizer and cause of allergic contact dermatitis (ACD) and occupational asthma (OA) (Bernstein *et al.*, 1993, NIOSH, 1994a, NIOSH, 1994b, Redlich and Karol, 2002, Lofgren *et al.*, 2003, NIOSH, 2004, Jan *et al.*, 2008, Engfeldt *et al.*, 2013). In an occupational setting, workers can be exposed and sensitized to MDI in liquid, vapor, or aerosol form or to MDI-coated particles such as wood dust (Woellner *et al.*, 1997), which may potentially lead to the development of MDI-associated OA (MDI-OA). To limit MDI exposure, the Occupational Safety and Health Administration (OSHA) currently sets the permissible exposure limit (PEL) for MDI at 0.2 mg/m^3 (0.02 ppm). In addition, the American Conference of Governmental Industrial Hygienists (ACGIH) has established a threshold limit value (TLV) of MDI at 0.005 ppm (0.05 mg/m^3) (ACGIH, 1999). Urine test methods using hydrolyzed 4,4'-Diaminodiphenylmethane (MDA) as a surveillance marker for MDI exposure have been suggested (Schutze *et al.*, 1995, Skarping and Dalene, 1995, Skarping *et al.*, 1995). However, the half-life of MDI metabolites in urine has been determined to be between 59–82 hours (Skarping *et al.*, 1995, Dalene *et al.*, 1997); therefore, these assays may only provide information on a worker with short term MDI exposure, rather than a longer-term cumulative exposure which may be more relevant in terms of the development of MDI sensitization. Detection of immune sensitization to MDI and/or early recognition of MDI-OA may be effective in slowing the progression of the disease (Wang and Petsonk, 2004); however, it can be difficult to recognize MDI-OA due to the lack of sensitive and reliable diagnostic tests for MDI exposure and sensitization.

In the clinical setting, physicians diagnose MDI-OA using patient histories of MDI exposure, though this is predictive in only 30–46% of cases (Malo *et al.*, 1991), or through an MDI specific inhalation challenge (SIC) which is expensive and poses possible health risks to the patient. The discovery of novel biomarkers for the early detection of MDI exposure and sensitization in workers is needed for early intervention to prevent subsequent MDI-OA. Significant effort has been focused on developing MDI-specific antibody-based tests for determination of MDI exposure and sensitization; however, these biomarkers are not sensitive enough to be used in a clinical setting (Keskinen *et al.*, 1988, Cartier *et al.*, 1989, Wass and Belin, 1989, Tee *et al.*, 1998, Ott *et al.*, 2007, Budnik *et al.*, 2013). By using proteomic methods, Hur *et. al.* first identified that ferritin and transferrin levels in serum may serve as biomarkers for MDI-OA (Hur *et al.*, 2008); however, the serum ferritin and transferrin levels failed to serve as biomarker for another closely-related diisocyanate, toluene 2,4-diisocyanate (TDI) (Sastre *et al.*, 2010). In addition, Haenen S *et. al.* identified several candidate biomarkers such as hemopexin for TDI-OA using a mouse model; however, these biomarkers for TDI-OA were not validated in humans (Haenen *et al.*, 2010, Haenen *et al.*, 2012, Haenen *et al.*, 2014). Other serological biomarkers such as matrix metalloproteinases-9 (MMP-9), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) have also been suggested as biomarkers for TDI-OA (Kim *et al.*, 2011); however, usefulness of these serum cytokines as markers for MDI-OA has yet to be demonstrated. Given the limited success of previous attempts to determine immunological, serological, and protein biomarkers for diagnosing MDI-OA, development of state-of-the art novel

techniques and classes of biomarkers to identify early MDI exposure and sensitization is needed.

MicroRNAs (miRs) are single-stranded RNA molecules ranging from 19 to 24 nucleotides in length with the ability to regulate diverse cellular processes through post-transcriptional regulation of target gene expression. The functional role of miRs in disease pathogenesis is an emerging field of study, and miRs are being utilized for disease diagnosis, prognosis, and evaluation of treatment response (Mendell and Olson, 2012, Srinivasan *et al.*, 2013). miRs may function by suppressing translation of target genes or by causing target messenger RNA (mRNA) degradation through imperfect binding to the 3' untranslated region (UTR) (Bartel, 2009). In addition to binding to the 3'UTR, miRs may be capable of binding to 5'UTRs, exons of mRNA and even DNA elements (Lytle *et al.*, 2007, Orom *et al.*, 2008, Place *et al.*, 2008, Fang and Rajewsky, 2011, Zhou and Rigoutsos, 2014). In some reports, miRs have been shown to upregulate target gene expression (Vasudevan *et al.*, 2007, Orom *et al.*, 2008, Cordes *et al.*, 2009, Lin *et al.*, 2011, Truesdell *et al.*, 2012). The majority of miRs are found in the intracellular region; however, many miRs may be found in the extracellular environment, including serum, plasma and other biological fluids (Valadi *et al.*, 2007, Chen *et al.*, 2008, Weber *et al.*, 2010, Arroyo *et al.*, 2011). Extracellular miRs commonly bind with RNA-binding proteins (RBPs), high-density lipoprotein particles (HDL) or are enclosed within lipid vesicles (exosomes, microvesicles, etc.) in the extracellular environment (Arroyo *et al.*, 2011, Turchinovich *et al.*, 2011, Vickers *et al.*, 2011). These extracellular miRs are reported to be relatively stable compared to other RNA species (Chen *et al.*, 2008, Mitchell *et al.*, 2008, Turchinovich *et al.*, 2011). Given their relatively high stability, and the fact that their expression levels have been associated with multiple disease development processes, extracellular miRs are considered to be good molecular candidates for biomarkers of disease. Circulating miRs, a subset of these extracellular miRs, are emerging as a novel class of minimally invasive biomarkers in different diseases including cancers (Mitchell *et al.*, 2008, Kosaka *et al.*, 2010), cardiovascular diseases (Gupta *et al.*, 2010, Tijsen *et al.*, 2012, Xu *et al.*, 2012), diabetes (Guay and Regazzi, 2013), and other diseases. Recent research on circulating miRs has mainly focused on their association with cancer and other diseases. Until recently, few reports focused on asthma-associated circulating miRs have been published (Wang *et al.*, 2015, Kho *et al.*, 2016, Panganiban *et al.*, 2016, Davis *et al.*, 2017, Milger *et al.*, 2017). Currently, there is no published research on MDI exposure associated circulating miRs, and we hypothesize that circulating miRs can be identified and used for detection of MDI exposure.

This report is focused on characterizing the response of circulating miRs to MDI exposure using a murine model. MDI exposure was performed dermally, via nose-only inhalation, and via nose-only challenge following dermal exposure/sensitization. Up/down-regulation patterns of mmu-miRs-183-5p, 206-3p and -381-3p were found consistently across three experimental murine models suggesting their putative roles as novel biomarkers for MDI exposure.

Clinical significance:

MDI exposure is a cause of occupational asthma. Early detection of MDI exposure/sensitization and timely removal of workers from exposure to MDI are essential for prevention of MDI-OA disease progression. The upregulation of circulating miR-183-5p, and downregulation of circulating miRs-206-3p and -381-3p can potentially be used to detect MDI exposure and/or sensitization.

Materials and methods

Chemicals and Reagents

HPLC grade acetone, 3Å molecular sieve (4–8 mesh), and 98% 4,4'-methylene diphenyl diisocyanate were acquired from Sigma-Aldrich (St. Louis, MO). Fatal-Plus® (sodium pentobarbital) euthanasia solution was acquired from Vortech Pharmaceuticals, Ltd. (Dearborn, MI). Dry acetone was prepared by incubating 10 ml HPLC grade acetone on 3 Å molecular sieve for a minimum of 24 hours to adsorb water.

Animals

Female BALB/c mice, 6–8 week old, were purchased from Taconic (Germantown, New York). Mice were acclimated at least for 5 days before being randomly assigned into different treatment groups. Mice were housed in ventilated plastic cages with hardwood chip bedding at 5 animals per cage. Each animal cage was enriched with a section of polyvinyl chloride (PVC) pipe (1.5" O.D. × 6") to acclimate animals to the nose-only restraint device. Further acclimation to the restraint device was performed by three stays of increasing duration (15 minutes, 30 minutes, 60 minutes) on three consecutive days in the restraint device. Device acclimation was well-tolerated. An NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and tap water were administered *ad libitum*. Housing facilities were maintained at 68–72 °F and 36–57% relative humidity with a 12-hour light-dark cycle. All animal experiments were performed in the AAALAC, International-accredited National Institute for Occupational Safety and Health animal facility in accordance with an institutionally-approved animal care and use protocol (Protocol # 16-JH-m-009).

MDI exposure

MDI dermal exposures were performed on groups of 5 mice by applying a single dose of 1% (w/v) MDI in dry acetone or dry acetone only (vehicle control; Ctl) on the dorsal surface of each ear (25 μl per ear) daily on 3 consecutive days. Mice were euthanized, ear thickness was measured using a caliper, ears and blood were collected 24 hours after final MDI dermal exposure. To verify candidate circulating miRs identified from miRCURY™ miRs qPCR Profiling Service, additional groups of 5 mice were dermally exposed to 1% MDI or vehicle Ctl. MDI aerosol exposures were performed on groups of 5 mice by exposing the animals, via a nose-only inhalation exposure system (NOIES) to MDI aerosol or pure house air (Control) for 1 hour as previously described (Hettick *et al.*, 2018). For each 1 hour aerosol exposure, the total of 4580 ± 1497 μg/m^3 of MDI concentrations were achieved and maintained. About half of the total MDI aerosol (2243 ± 903.8 μg/m^3) generated during the 1 hour exposure were particles less than 3.0 μm in diameter. MDI dermal exposure/aerosol

challenge was performed on groups of five mice by applying 25 μl 1% MDI/acetone (w/v) dose on the dorsal surface of each ear on days 1, 2, 3, 14, 15, and 16. The second dermal exposure on days 14, 15, and 16 served as a booster exposure to achieve a potential better sensitization response as previously described (Vanoirbeek *et al.*, 2004, Selgrade *et al.*, 2006). On day 21, the animals were nose-only exposed to MDI aerosol using the NOIES for 1 hour, similar to the aerosol-only group. For both MDI aerosol exposures and MDI dermal exposure/aerosol challenge groups, mice were euthanized at 4 hours and 24 hours after MDI aerosol exposure.

Euthanasia, serum, tissue collection, and processing

Animals were euthanized via intraperitoneal injection of sodium pentobarbital. Following a non-reflexive response to a toe pinch test, exsanguination was performed via cardiac puncture and blood was placed into serum collection tubes (Blood Collection Microtainer Tube with Serum Separator, Becton Dickinson, San Jose, CA), centrifuged, and serum was collected and stored at −80 °C for subsequent RNA analysis. Ears from MDI dermal exposure experiments were collected and stored at −80 °C for RNA isolation and subsequent gene expression analysis.

Tissue RNA isolation, reverse transcription, and Real-Time PCR

MDI or dry-acetone exposed ears were processed for total RNA isolation using a Tissue Lyser II (Qiagen, Hilden, Germany) in *mirVana*™ miR lysis/binding buffer (Thermo Fisher Scientific). Tissue total RNA was isolated using *mirVana*™ miR Isolation Kit (Thermo Fisher Scientific). The concentration and purity of the RNA was determined using a ND-1000 spectrophotometer (Thermo Fisher Scientific). 200 ng of total RNA was subjected to first strand cDNA synthesis using a High-Capacity cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's protocol on a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY). For analysis of mRNA expression, TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific), cDNA, and mouse-specific mRNA TaqMan® assays were combined and PCR was performed according to manufacturer's protocol. Assays used in this study include: Il-1β (Mm00434228_m1), Il-2 (Mm00434256_m1), Il-4 (Mm00445259_m1), Il-5 (Mm00439646_m1), Il-6 (Mm00446190_m1), Il-13 (Mm00434204_m1), Tnf-α (Mm00443258_m1), Inf-γ (Mm01168134_m1), Fcer1a (FcεRI) (Mm00438867_m1) and B2m (Mm00437762_m1). Real-time PCR was performed on an ABI PRISM 7500 (Thermo Fisher Scientific) with the following cycling conditions: 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. B2m served as the endogenous reference control. The relative expression levels of mRNAs were calculated using the comparative $^{\Delta\Delta}$Ct method as previously described (Lin *et al.*, 2011, Sharma *et al.*, 2014, Lin *et al.*, 2015).

Circulating miRs qPCR array profiling assay

The MDI dermal exposure mouse serum was used to characterize candidate circulating miRs for MDI exposure biomarker identification. Serum samples were sent to Exiqon (Vedbaek, Denmark) for the miRCURY™ miRs qPCR Profiling Service to profile circulating miRs. A total of 310 miRs were detected in both control and MDI exposed samples by miRCURY™

miRs qPCR Profiling Service. The heat map for all 310 miRs were generated by the online analysis tool, CIMminer (https://discover.nci.nih.gov/cimminer/).

Verification of candidate circulating miRs

200 μL sera from independent, separate groups of MDI-dermal exposure, MDI-aerosol exposure, and MDI dermal exposure/MDI-aerosol challenge mice were used for total RNA isolation using *mirVana*™ PARIS™ Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol for liquid samples. The concentration and purity of the RNA were determined using an ND-1000 spectrophotometer (Thermo Fisher Scientific). To synthesize cDNA for miRs assays, 10 ng of serum total RNA from each treatment was subjected to reverse transcription (RT) using a TaqMan® miR Reverse Transcription Kit (Thermo Fisher Scientific) and specific TaqMan RT primers included in the miR assays following the manufacturer's protocol. Specific TaqMan miR assays (Thermo Fisher Scientific) were acquired: mmu-miR-16-5p (Assay ID #000391), mmu-miR-30d-3p (#002305), mmu-miR-127-3p (#000452), mmu-miR-153-3p (#001191), mmu-miR-181a-1-3p (#000516), mmu-miR-183-5p (#002269), mmu-miR-192-3p (#002272), mmu-miR-206-3p (#000510), mmu-miR-381-3p (#000571), mmu-miR-433-3p (#001028), and mmu-miR-744-3p (#002325). RT was performed on a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) with the following cycling conditions: 16 °C for 30 minutes, 42 °C for 30 minutes, followed by a final step of 85 °C for 5 minutes to inactivate the reverse transcriptase. To generate enough miR cDNA template for following real-time PCR reaction, the cDNA was pre-amplified using specific miR TaqMan assays and a PreAmp Master Mix (Thermo Fisher Scientific) following the manufacturer's instructions. The PreAmp primer pool consisted of 0.2× TaqMan primers specific for each of the candidate miRs. The pre-amplification cycling conditions were as follows: 95 °C for 10 min followed by 14 cycles of 95 °C for 15 seconds and 60 °C for 4 minutes.

After the pre-amplification step, the products were diluted 200-fold with RNase-free water and served as template for real-time PCR reaction. Real-time PCR was performed as described above. Circulating mmu-miR-16-5p was previously reported as being constantly expressed in serum (Ng *et al.*, 2009, Tanaka *et al.*, 2009, Wang *et al.*, 2009, Zhu *et al.*, 2009) and was used as an endogenous control in this study. The relative expression levels of miRs were calculated using the comparative $^{\Delta\Delta}$Ct method as described previously (Lin *et al.*, 2011, Sharma *et al.*, 2014, Lin *et al.*, 2015).

Candidate circulating miRs *in silico* pathway analysis

Predicted targets of candidate circulating miRs were obtained from 7 *in silico* algorithms, including DIANA-microT (Maragkakis *et al.*, 2009a, Maragkakis *et al.*, 2009b), miRanda (Enright *et al.*, 2003, Betel *et al.*, 2008), mirBridge (Tsang *et al.*, 2010), PicTar (Krek *et al.*, 2005), PITA (Kertesz *et al.*, 2007), rna22 (Miranda *et al.*, 2006), and TargetScan (Lewis *et al.*, 2005, Grimson *et al.*, 2007, Friedman *et al.*, 2009). Furthermore, two experimentally-validated databases were queried, TarBase (Papadopoulos *et al.*, 2009) and miRecords (Xiao *et al.*, 2009) using the web-based tool miRsystem (http://mirsystem.cgm.ntu.edu.tw/) (Lu *et al.*, 2012). Pathway enrichment analysis of candidate miRs target genes was conducted using

the miRsystem online tool with three pathway databases: KEGG (Kanehisa *et al.*, 2008), BioCarta (Nishimura, 2001), and Reactome (Matthews *et al.*, 2009).

Statistical analysis

Data were analyzed using either the unpaired *t*-test (two-tailed) in MDI-dermal exposure experiments (Figures 1&3) or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison *ad hoc* post-test in MDI-aerosol exposure and MDI-dermal exposure/MDI-aerosol challenge experiments (Figures 4&5). Statistical analyses were performed in GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA). The empirical *P*-values of each identified enriched pathway were determined by using the default settings of miRsystem (Lu *et al.*, 2012). Differences were considered significant when the analysis yielded $P<0.05$.

Results

Examination of immunological responses, skin irritancy and sensitization potential of MDI dermal exposure

To confirm that exposure to MDI would result in possible inflammation, irritation and sensitization of the animals, 1% MDI (in dry acetone, w/v) was painted on each ear of randomly grouped BALB/c mice for 3 consecutive days followed by collection of ears and blood on day 4 (Figure 1A). Ears were assayed for swelling and were used for RNA isolation. Expressions of inflammatory cytokines, Th1 and 2 cytokines, and the high affinity IgE receptor were measured by qRT-PCR, (Figure 1B-K). This 1% MDI dosing regimen was selected because 1% TDI dermal exposure was proven sufficient to induce sensitization (Anderson *et al.*, 2014). Furthermore, Selgrade *et. al.* reported that 1% MDI dermal exposure on the shaved back of BALB/c mice was sufficiently to induce serum IgE and Th2 cytokines (Selgrade *et al.*, 2006). Average ear swelling was significantly increased four days following 1% MDI exposure (Figure 1B). Inflammatory cytokines (Il-1β, Il-6, and Tnf-α,) mRNA levels were significantly increased in the ear four days following 1% MDI exposure (Figure 1 C-E), indicating that dermal MDI exposure may cause an inflammation response. To examine whether this dermal exposure regimen may result in possible sensitization responses, we measured Th1 (Il-2 and Inf-γ), Th2 cytokines (Il-4, -5, and -13) and high affinity IgE receptor mRNA levels in the ear. Inf-γ mRNA level was significantly upregulated 42.7-fold (Figure 1G) whereas Il-13 mRNA level was significantly increased 4.46-fold (Figure 1J) in the ear four days following 1% MDI exposure compared to vehicle control. The expression of Th1 Il-2 mRNA (Figure 1F) and Th2 Il-4 mRNA (Figure 1H) were not detectable in either MDI or vehicle control exposed ears whereas the Th2 Il-5 mRNA was not changed (Figure 1I). Given that the expression level of the α subunit of the high-affinity IgE receptor (Fc epsilon RI; FcεRI) in human mast cells and basophil has been found to be directly correlated with serum IgE levels (Gomez *et al.*, 2007), and the presence of MDI-specific IgE or increase of total IgE level indicates that there is systemic sensitization to MDI (Wisnewski *et al.*, 2011, Pollaris *et al.*, 2016), we used FcεR1 mRNA expression in the ear as a measure of possible sensitization. FcεR1 mRNA was significantly increased 13.56-fold four days following 3 consecutive days of 1% MDI exposure (Figure 1K). These data suggest that dermal exposure to 1% MDI induces significant immunological responses in the ear, and it may cause sensitization to MDI.

Circulating miR profiling and verification in murine model

To identify potential circulating miRs as biomarkers for MDI dermal exposure, we used miRCURY™ miRs qPCR Profiling Service (Exiqon) to profile serum miR changes between dermal MDI-exposed and control mice. Of the total 752 miRs profiled, 310 miRs were detected in both MDI-exposed and control mice (Figure 2). To refine the list of candidate miRs, we identified the top 20 differentially expressed miRs between the two groups (Table 1). Of these 20 miRs, we constrained our analysis to the 10 miRs that have human homologs. We then independently verified the expression of these 10 miRs in-house using TaqMan® miR stem-loop qRT-PCR assays on another separate groups of mice dermally exposed to MDI and vehicle control (Figure 3A). Compared with serum RNA isolated from control mice, mmu-miR-183-5p was upregulated 82.1-fold (Figure 3B), whereas mmu-miR-206-3p and mmu-miR-381-3p were downregulated 1.59-fold (Figure 3C) and 6.31-fold (Figure 3D), respectively. Of the putative differentially expressed miRs identified in the miRCURY™ miRs qPCR Profiling Service, mmu-miRs-127, -192-3p, -181a-1-3p, -433-3p and -744-3p (Figure 3E-I) failed verification (by RT-PCR) between MD-Iexposed and control mice. Furthermore, mmu-miRs-30d and -153-3p were not detected in either MDI-exposed or control mice (Figure 3J and K). Moving forward, we considered only circulating mmu-miRs-183-5p, -206-3p and -381-3p as potential biomarkers for dermal MDI exposure.

Circulating miR validation in nose-only aerosol exposure murine model

Occupational exposure routes to MDI are thought to be mostly through inhalation and dermal contact (NIOSH, 2004). At room temperature, MDI is not volatile (vapor pressure is approximately at 10^{-5} mm Hg). However, during application, MDI is frequently heated and/or aerosolized via a spray gun, thus generating respirable vapor and/or aerosols. To mimic MDI occupational airway aerosol exposure, we have developed a nose-only aerosol exposure mouse model (Hettick *et al.*, 2018). We further determined whether the circulating mmu-miRs-183-5p, -206-3p, and -381-3p expression changes in response to aerosol exposure, as it does for dermal exposure. Compared to mice exposed to house air only, mmu-miR-183-5p was upregulated 16.47-fold in serum collected at 4 hours (4h) post MDI aerosol exposure and 36.12-fold in serum collected at 24 hours (24h) after MDI aerosol exposure (Figure 4 A), whereas mmu-miR-206-3p was downregulated 10.17-fold at 4h and 3.29-fold at 24h post MDI exposure (Figure 4 B). In addition, serum mmu-miR-381-3p levels were downregulated 7.94-fold at 4 h and 3.65-fold at 24h after MDI-aerosol exposure compared to air only control (Figure 4C). These results suggest that circulating mmu-miRs-183-5p, -206-3p and -381-3p can also be used as putative biomarkers for detection of short term airway exposure to MDI.

Dermal exposed MDI aerosol challenge murine model

MDI dermal exposure followed by MDI inhalation has been associated with MDI-OA attack (Rattray *et al.*, 1994, Petsonk *et al.*, 2000). To investigate whether candidate miRs can serve as MDI exposure biomarkers over a longer term (21 days), as well as in an industrially-relevant exposure model, we developed a MDI-dermal exposure followed by MDI-aerosol challenge murine model suggestive of OA exposure (Figure 5A). Examination of candidate miRs in the house-air exposed control mice (D−/A− vs. D+/A−) failed to demonstrate a

change in mmu-miR-183-5p level (Figure 5B) but caused 15.6-fold and 6.15-fold downregulation of serum mmu-miRs-206-3p and -381-3p (Figure 5C&D), indicating that only downregulation of both circulating miRs-206-3p and -381-3p may serve as biomarker for long term repeated dermal MDI exposure. Circulating mmu-miR-183-5p was upregulated 2.58-fold at 4hr following aerosol challenge (D+/A+) but returned to control levels 24hr after compared to D−/A− control (Figure 5B). Circulating mmu-miR-206-3p level was downregulated 23.33-fold at 4hr and 18.47-fold 24hr after MDI aerosol exposure (D+/A+) compared to control (D−/A−) (Figure 5C). In addition, circulating mmu-miR381-3p levels were downregulated 5.22-fold at 4hr and 2.45-fold at 24hr after MDI exposure (D+/A+) compared to control (D−/A−) (Figure 5D). The non-responsiveness of circulating mmu-miR-183-5p from repeated MDI-dermal exposure (D−/A− vs. D+/A−, Figure 5B) may be due to desensitization of the upregulation of circulating mmu-miR-183-5p or the result of rapid clearance from circulation through an unknown mechanism(s). This putative mechanism(s) of non-responsiveness of circulating mmu-miR-183-5p from repeated dermal MDI exposure may be the cause of the comparatively small 2.58-fold upregulation at 4hr following MDI aerosol challenge (D+/A+_4hr, Figure 5B) versus a higher 16.47-fold upregulation at 4hr following MDI aerosol only exposure (Figure 4A). Similarly, this putative mechanism(s) may also be the cause for no observed upregulation of circulating mmu-miR-183-5p at 24hr following MDI aerosol challenge (D+/A+_24hr, Figure 5B). The rapid upregulation of circulating mmu-miR-183-5p after MDI dermal exposure only (Figure 3B) and MDI aerosol exposure only (Figure 4A) represents the early response for acute MDI exposure. Both circulating mmu-miRs-206-3p and -381-3p were downregulated in MDI dermal exposure (Figure 3C&D) and were rapidly downregulated in MDI aerosol exposure (see MDI-4h vs. Air; Figure 4B&C); therefore, both rapid upregulation of circulating mmu-miR-183-5p and rapid downregulation of circulating miRs-206-3p and -381-3p may serve as biomarkers for acute (~4hr post-exposure) MDI exposure. In addition, downregulation of circulating miRs-206-3p and -381-3p may serve as good biomarkers for detection of MDI aerosol exposure on longer time scales (24 hours +).

Candidate circulating miR targets and pathway analysis

To explore the biological mechanisms underlying the roles of identified circulating miRs in relation to MDI exposure and MDI-OA, we performed target identification of human homolog miRs, hsa-miR-183-5p, hsa-miR-206-3p, and hsa-miR-381-3p on miRsystem (Lu *et al.*, 2012). *In silico* analysis of target prediction returned 446 target genes for hsa-miR-183-5p (Supplemental Table S1), 699 target genes for hsa-miR-206 (Supplemental Table S2), 622 target genes for hsa-miR-381-3p (Supplemental Table S3). 89 genes were co-targeted by both hsa-miRs-206 and 381-3p (Supplemental Table S4), and 1142 genes can be targeted by either hsa-miR-206 or hsa-miR-381-3p (Supplemental Table S5).

Pathway analysis against KEGG, Reactome, and BIOCARTA databases showed a total of 79 pathways are potentially impacted by hsa-miR-183-5p (Table 2). Twelve enriched pathways had ≥8 potential genes that potentially can be targeted by hsa-miR-183-5p and the majority of the pathways were related to human diseases, such as HIV-1 relative pathways, prion disease, Type I diabetes mellitus, Huntington’s disease, and long-term depression. Pathway

analysis for potential co-targets of hsa-miR-206 and -381-3p revealed 38 pathways. Among those pathways, five of the pathways were related to the immune system, such as Fc gamma R-mediated phagocytosis, NFAT pathway, chemokine signaling pathway, FMLP pathway, and leukocyte transendothelial migration (Table 3). Fourteen of these pathways had ≥9 potential genes that could potentially be co-targetted by both hsa-miR-206, and -381-3p. The predicted co-targets also were involved in the signaling transduction pathways, such as signaling by PDGF, downstream signal transduction, G alpha (Q) signaling events and signaling to Erks.

Discussion

Circulating miRs have been used as novel minimally invasive biomarkers for a variety of diseases since Mitchell *et al.* first reported that circulating miRs had potential for detection of different cancers in 2008 (Mitchell *et al.*, 2008). Since then, few studies have focused on identifying circulating miRs in asthma until recently (Wang *et al.*, 2015, Kho *et al.*, 2016, Panganiban *et al.*, 2016, Davis *et al.*, 2017, Milger *et al.*, 2017). The use of circulating miRs for detection of dNCO exposure and dNCO-associated OA have not been previously reported. To our knowledge, this study is the first report to determine circulating miR biomarkers for detection of MDI exposure in murine models. We identified that circulating mmu-miRs-183-5p, -206-3p and -381-3p levels were either upregulated or downregulated after MDI exposure and determined that the up/down-regulation patterns of these three circulating miRs may potentially serve as biomarkers for detection of MDI exposure.

Recent studies identified circulating hsa-miRs-15a, -16, -21, -27a, -29c, -30d-5p, -125b, -126, -133b, -206, -223, -299-5p, -342, -425, -1260a, and -3162-3p may serve as novel biomarkers for allergic rhinitis or asthma diagnosis (Wang *et al.*, 2015, Kho *et al.*, 2016, Panganiban *et al.*, 2016, Davis *et al.*, 2017, Milger *et al.*, 2017). In those reports, there is not a single circulating miR identified that was repeatedly identified between studies. In our study, the murine homolog to circulating hsa-miR-206 was identified as a component of a putative biomarker pattern for MDI exposure. Expression of hsa-miR-206 has been shown to be elevated in patients with allergic rhinitis, but similar in patients with asthma and heathy subjects (Panganiban *et al.*, 2016). This study shows that the level of miRs-206 and -381-3p are decreased in mouse after MDI exposure, indicating that the MDI-triggered downregulation of both circulating miRs-206 and -381-3p is specific, and may be used to distinguish MDI exposure from other conditions.

The physiological roles that miRs-183-5p, -206-3p, and -381-3p play in association with asthma and MDI exposure are currently unknown and are worthy of subsequent functional studies. For this study, we used pathway enrichment assays to predict the functional roles in association with MDI exposure. Many pathways enriched in potential genes regulated by hsa-miR-183-5p were associated with HIV-related diseases; however, the single most significant pathway identified is the neuronal system. One of the protein targets of hsa-miR-183-5p (Table 2, and Supplemental Table S1) identified in the neuronal system is the potassium calcium-activated channel subfamily M regulatory beta subunit 1 (KCNMB1), which is associated with lung diseases such as COPD and asthma (Seibold *et al.*, 2008, Cao *et al.*, 2014). In the airway smooth muscle (ASM), the increase of intracellular calcium

concentration triggered by muscarinic acetylcholine receptors activation cause ASM contraction, and consequently, an asthma attack. The large conductance, Ca^{2+} and voltage-dependent K^+ (BK) channels decrease intracellular calcium concentration leading to ASM relaxation; therefore, the BK channel proteins become potential treatment targets for COPD and asthma (Pelaia *et al.*, 2002). The BK channels are composed of a pore forming α-subunit which is encoded by KCNMA1 and a regulatory β-subunit which is encoded by KCNMB1. Interestingly, hsa-miR183-5p was elevated and has been shown to downregulate KCNMB1 expression in COPD lung tissues (Cao *et al.*, 2014). In our study, we identified that hsa-miR-183-5p was elevated after MDI exposure, and subsequent studies should examine if MDI exposure-related elevation of hsa-miR-183-5p will downregulate KCNMB1 in the lung ASM, leading to accumulation of intracellular calcium, smooth muscle contraction, and subsequent asthmatic symptoms.

Among the several different pathways that are enriched in genes potentially regulated by both hsa-miRs-206-3p and -381-3p, we found that the immune related pathways were highly enriched. Interestingly, all enriched immune related pathways involve calcium related signaling. One of the important calcium related signaling pathways is the NFAT (Nuclear Factors of Activated T-Cells) transcription factors activation signaling pathways. NFAT transcription factors were originally described as important transcription regulators in naive T cells and differentiated effector T cells (Macian, 2005), but have been found to be expressed in many diverse cell types in the immune system including dendritic cells (Goodridge *et al.*, 2007, Zanoni *et al.*, 2009), mast cells (Monticelli *et al.*, 2004, Klein *et al.*, 2006), B-Cells (Berland and Wortis, 2003, Winslow *et al.*, 2006), NK (Natural Killer) T cells (Lazarevic *et al.*, 2009), and other cell types. The activation of NFATs is induced by receptor-coupled calcium signaling, which includes activation of calcium binding calmodulin and activation of the calmodulin-dependent phosphatase, calcineurin. Activated calcineurin dephosphorylates and activates cytosolic NFATs, which translocate into the nucleus and mediate gene expression (Feske *et al.*, 2003, Hogan *et al.*, 2003). Associated with other transcription factors such as AP-1 (Macian *et al.*, 2001), NFATs can mediate expression of a number of immunologically important genes, including Th1 type cytokines: IL-2 and INFγ (Peng *et al.*, 2001), Th2 type cytokines: IL-4, IL-5, and IL-13 (Zhang *et al.*, 1999, Burke *et al.*, 2000, Macian *et al.*, 2000), and inflammatory relative genes: IL-3, GM-CSF, and TNFα (Cockerill *et al.*, 1995, Bert *et al.*, 2000, Macian *et al.*, 2000). Cell-surface proteins such as CD40L, FasL and CTLA4 on immune related cells are also regulated by NFATs (Im and Rao, 2004, Macian, 2005). Dysregulation of NFAT signaling has been associated with asthma (Hodge *et al.*, 1996, Keen *et al.*, 2001, van Rietschoten *et al.*, 2001, Diehl *et al.*, 2002, Rengarajan *et al.*, 2002a, Rengarajan *et al.*, 2002b, Koch *et al.*, 2015). Our observation that the murine homologs of circulating hsa-miRs-206-3p and -381-3p were decreased after MDI exposure suggests the interesting hypothesis that calcium dependent NFAT signaling is activated in MDI exposed cells.

Anderson *et. al.* first reported upregulation of miRs-21, -22, -27b, -31, -126, -155, -210, and -301a from draining lymph node (dLN) of TDI dermally sensitized mice (Anderson *et al.*, 2014). In a follow-up mechanistic study, it was determined that miR-210 upregulation may inhibit regulatory T cell (T_{reg}) function during TDI sensitization (Long *et al.*, 2016). Given that TDI and MDI are both aromatic diisocyanates containing the same reactive moiety

(N=C=O) and that both are potent sensitizers, we would expect that MDI and TDI dermal exposure should cause similar miR responses. However, significant changes to expression were not observed for circulating miRs-21, -22, -27b, -31, -126, -155, -210, and -301a between MDI dermal exposed and control mice (Table 1 and data not shown). It is as yet unclear whether the differences observed between these results and those of Anderson *et al.* are due to differences in isocyanate (TDI vs. MDI), time point (4 days vs. 4/24 hr), or tissue type (draining lymph node vs. serum). Furthermore, detailed mechanistic understanding of how specific miRs are selected and released into circulation from the intracellular environment remains unclear. Evidence has shown that intracellular miRs can be released into extracellular environment through packaging into exosomes (Valadi *et al.*, 2007), microvesicles (Hunter *et al.*, 2008), apoptotic bodies (Zernecke *et al.*, 2009), binding to high density lipoprotein (HDL) (Vickers *et al.*, 2011) and AGO protein complex (Arroyo *et al.*, 2011, Turchinovich *et al.*, 2011). Certainly, circulating miRs reflect a systemic response to the chemical exposure rather than a tissue- or cell-specific response. Future studies are planned to investigate whether the up/down-regulation of miRs-183-5p, -206-3p, and -381-3p identified in current study are observed in the dLN cell population.

Conclusion

We identified that circulating mmu-miRs-183-5p, -206-3p and -381-3p are up/down-regulated after MDI exposure in murine models. Because these miRs are conserved in humans, the findings support the hypothesis that circulating miRs-183-5p, -206-3p and -381-3p may serve as easily obtainable, measurable, circulating biomarkers of MDI-exposure in workers. Given that miRs have been shown to have prognostic value in many diverse diseases, including cancers (Mitchell *et al.*, 2008, Ng *et al.*, 2009, Zhu *et al.*, 2009, Kosaka *et al.*, 2010, Freres *et al.*, 2016, Heishima *et al.*, 2017) and are involved in other inflammatory processes (Zampetaki *et al.*, 2010, Hromadnikova *et al.*, 2014, Xu *et al.*, 2014), future studies on the predictive value of these miRs, and their pathophysiological mechanisms in relation to MDI exposure/sensitization and MDI-OA is needed. Furthermore, these circulating miRs should be evaluated in exposed worker populations for validation as biomarkers of MDI exposure. Finally, pathway analysis of two downregulated miRs (miRs-206-3p and -381-3p) suggests that calcium-dependent NFAT signaling is a candidate pathway for functional studies on diisocyanate induced disease pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclosure Statement

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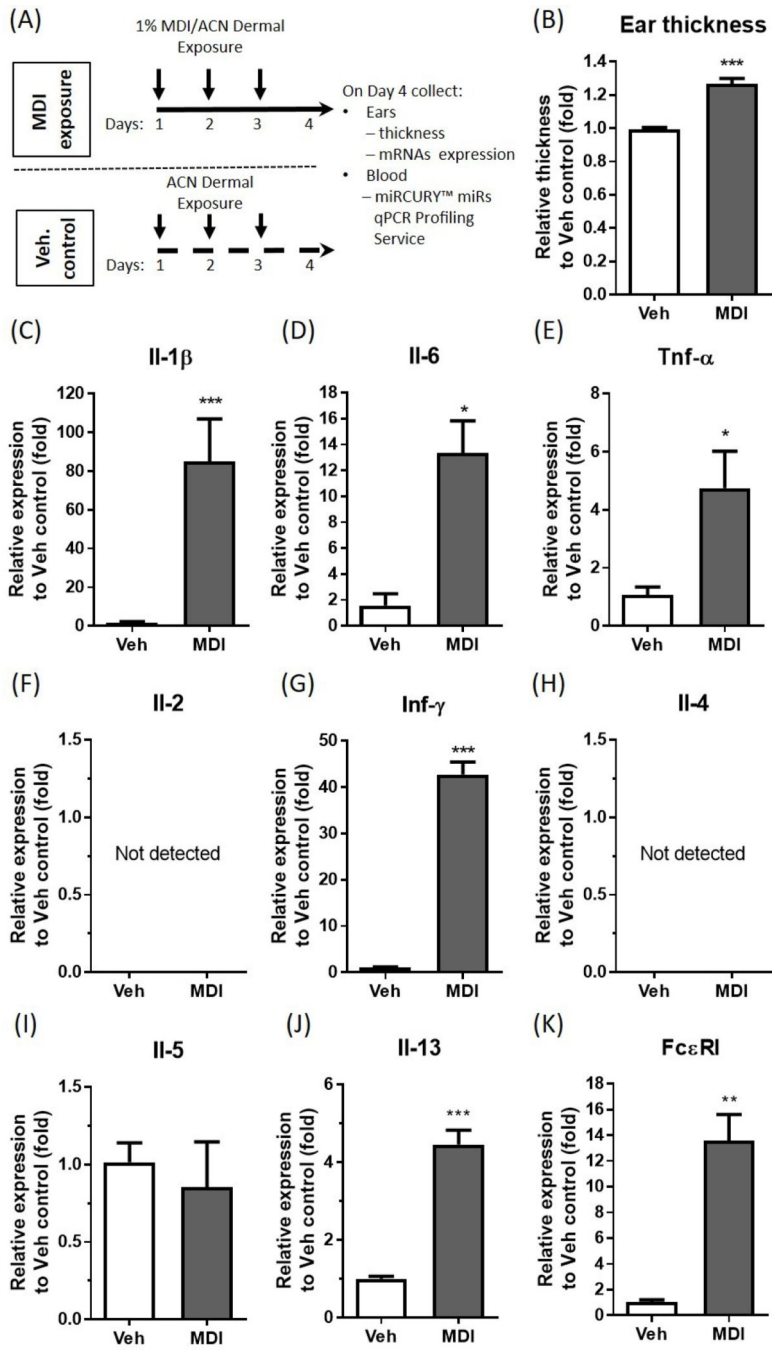

Figure 1. Examination of immunological responses, skin irritancy and sensitization potential of MDI dermal exposure.

Experimental timelines showing MDI exposed time points, sample collection and analyses (A). Relative ear thickness change as determined 4 days following 1% MDI/ACN exposure (B) (N=30; bars, s.e.m.). Ear mRNA expression of the inflammatory cytokines Il-1β (C), Il-6 (D), Tnf-α (E), Th1 cytokines Il-2 (F) and Inf-γ (G), Th2 cytokines Il-4 (H), Il-5 (I) and Il-13 (J) as well as high affinity IgE receptor, FcεRI (K) were determined 4 days following MDI exposure via RT-qPCR (N=3; bars, s.e.m.). MDI: 4,4'-methylene diphenyl diisocyanate; Veh: vehicle; ACN: acetone. (*P<0.05; **P<0.01; ***P<0.001).

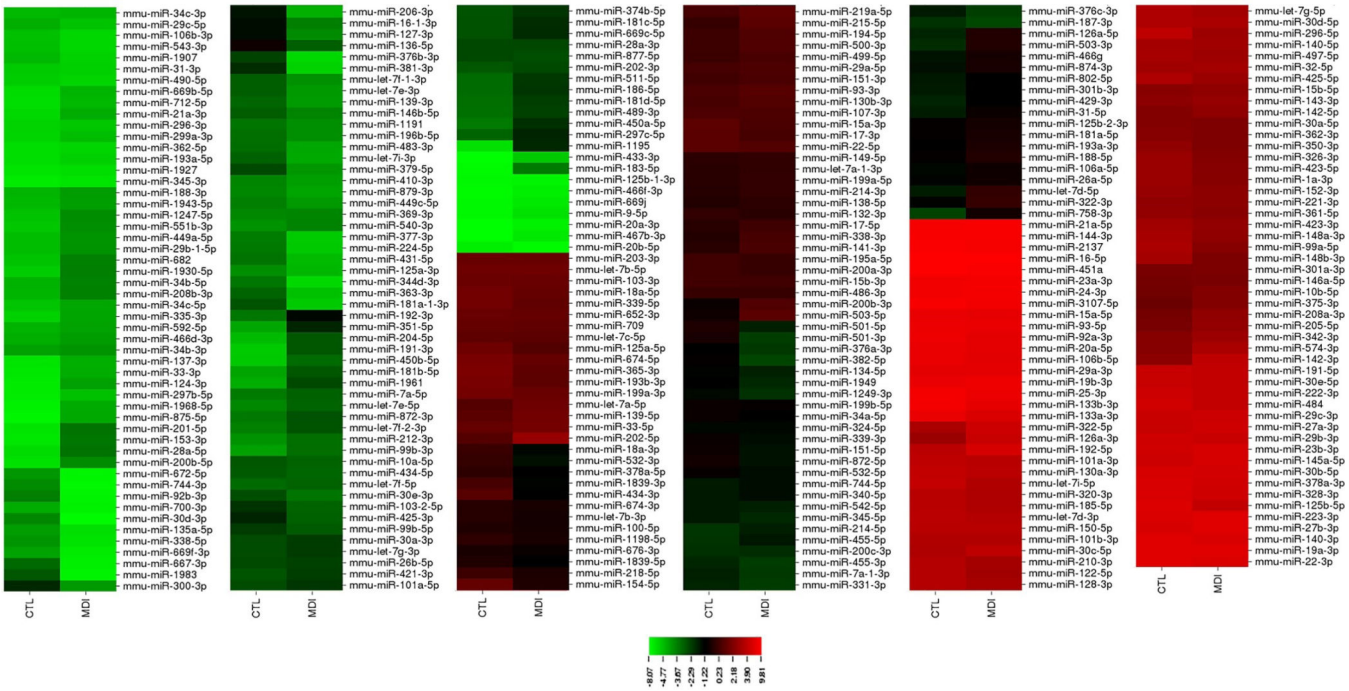

Figure 2. Heat map shows circulating miRs detected in both dermal MDI-exposed and vehicle control exposed mice.

Serum were collected from mice dermally exposed to either 1% MDI in dry acetone (MDI) or vehicle control (CTL), and sent to Exiqon in Denmark for miRCURY™ miRs qPCR Profiling Service to profile miR expressions. Total 310 miRs were detected in both MDI and control mice. The color scale shown at the bottom illustrates the relative expression level of a miR across all samples: red color represents an expression level above mean, green color represents expression lower than the mean. (Please see Figure 2 online at (https://doi.org/10.1080/1354750X.2018.1508308) for the color version).

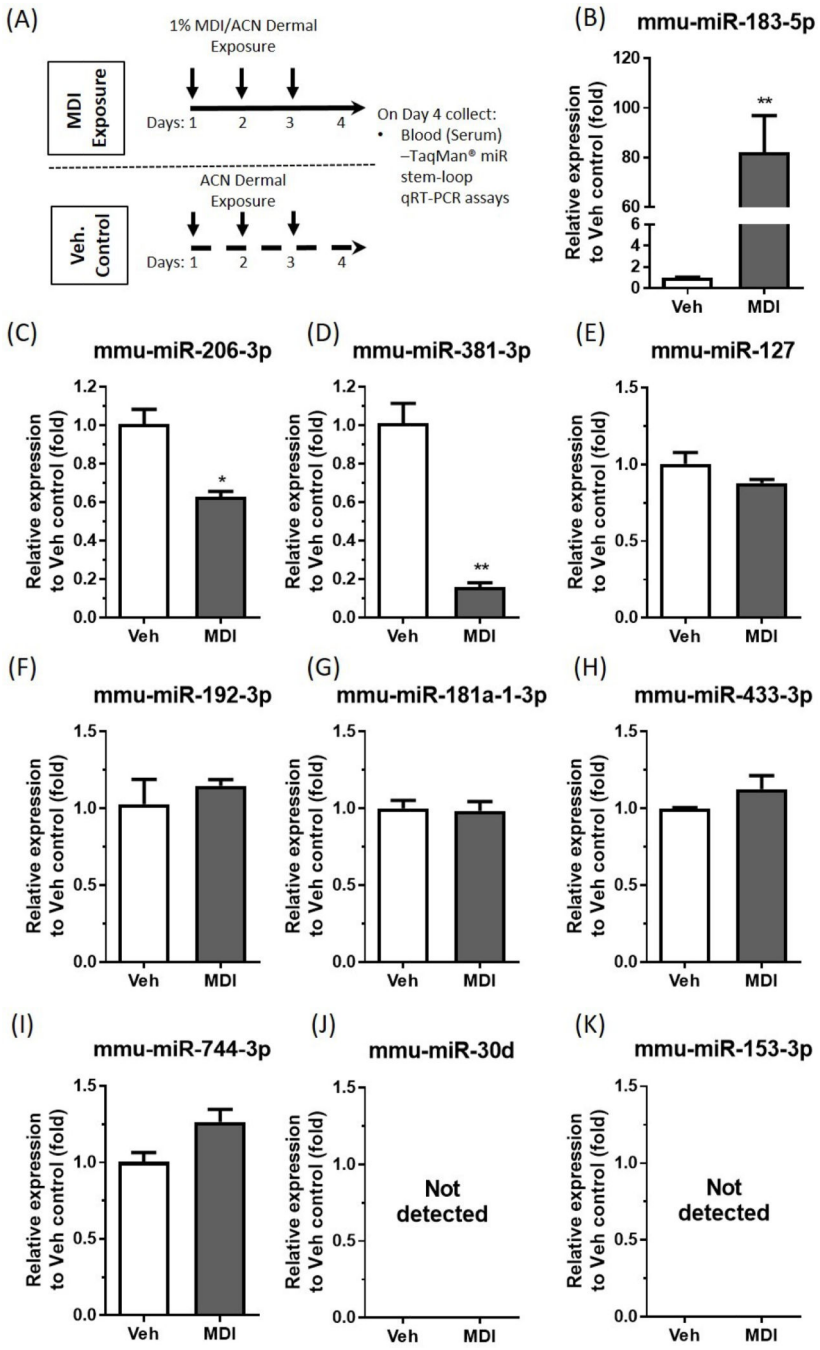

Figure 3. Verification of candidate circulating miRs by stem-loop qRT-PCR on MDI dermal exposed mice.

Experimental timelines showing MDI exposed time points, sample collection and analysis in additional separate groups of mice (A). Serum total RNA were isolated by *miRVana™ PARIS™* isolation kit, reverse transcribed, preamplified and subjected to TaqMan miR stem-loop qRT-PCR. Circulating miR expressions of candidate (B) mmu-miR-183-5p, (C) mmu-miR-206-3p, (D) mmu-miR-381-3p, (E) mmu-miR-127, (F) mmu-miR-192-3p, (G) mmu-miR-181a-1-3p, (H) mmu-miR-433-3p, (I) mmu-miR-744-3p, (J) mmu-miR-30d and (K) mmu-miR-153-3p changes were determined 4 days following 1% MDI/ACN exposure

(N=3; bars, s.e.m). MDI: 4,4'-methylene diphenyl diisocyanate; Veh: Vehicle; ACN: acetone. (*P<0.05; **P<0.01).

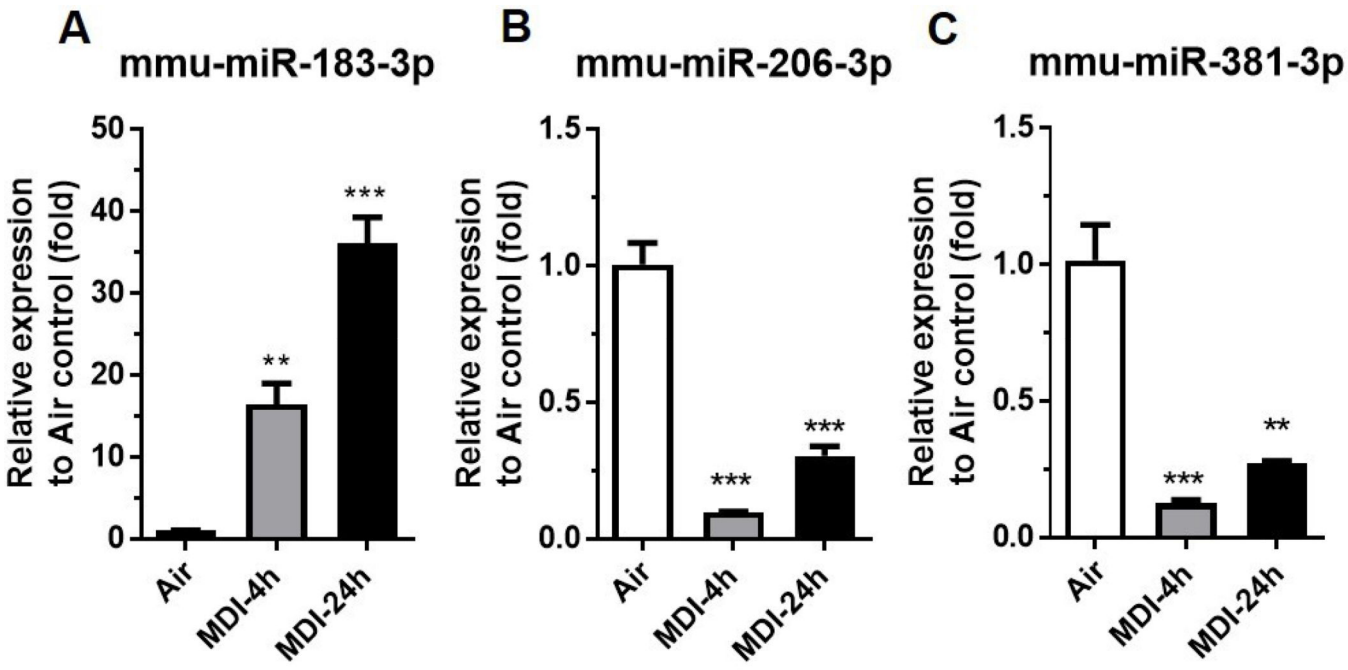

Figure 4. Verification of candidate serum miRs by stem-loop qRT-PCR on nose-only MDI-aerosol exposed mice.

Serum total RNA was isolated using the *miRVana™ PARIS™* isolation kit, reverse transcribed, preamplified and subjected to miR stem-loop qRT-PCR. Circulating miR expressions of candidate (A) mmu-miR-183-5p, (B) mmu-miR-206-3p, and (C) mmu-miR-381-3p were determined at 4 hours or 24 hours after 1 hour of nose-only MDI-aerosol exposure (N=3; bars, s.e.m). MDI: 4,4'-methylene diphenyl diisocyanate. (***P*<0.01; ****P*<0.001).

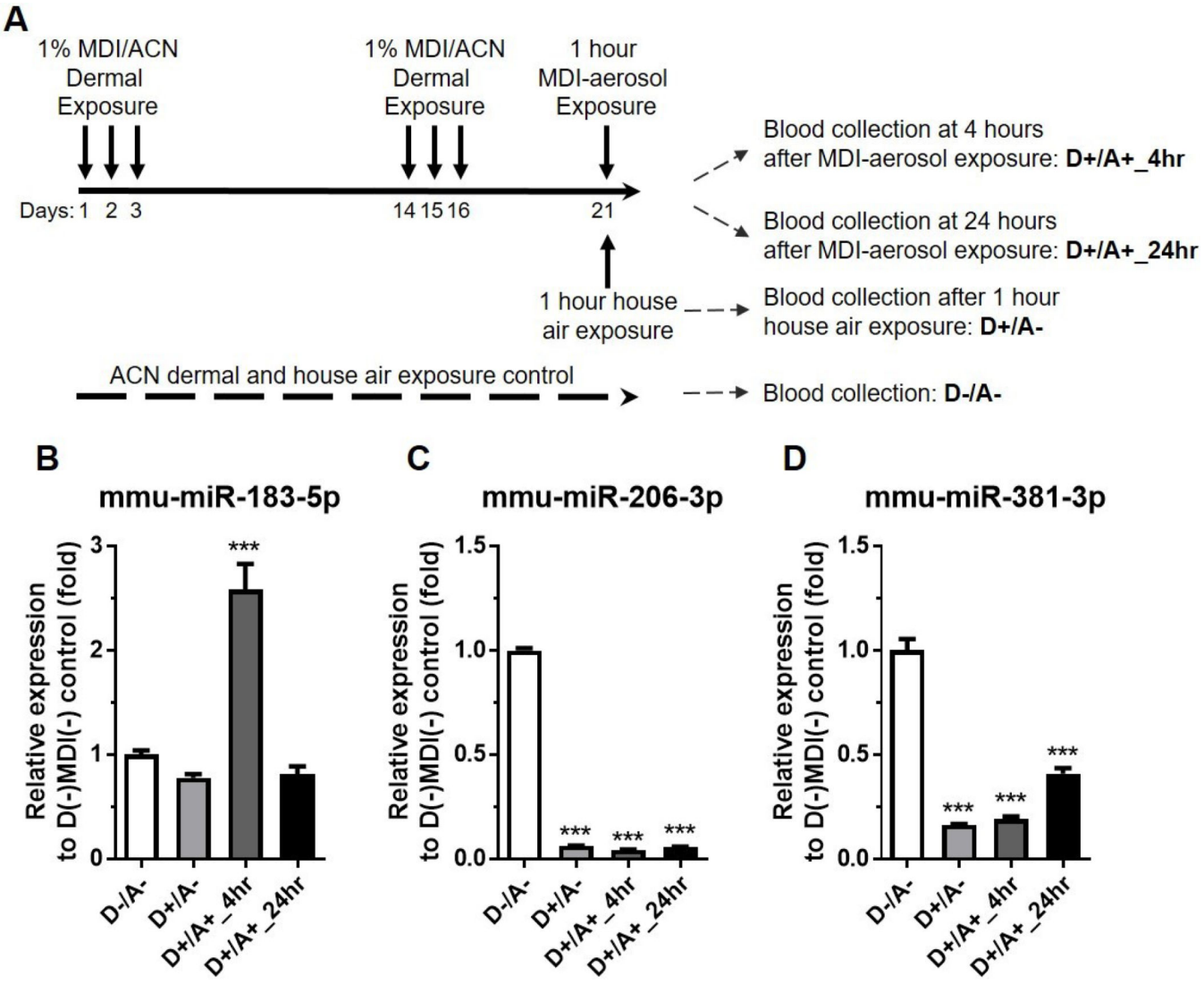

Figure 5. Determination of candidate circulating miRs in dermal exposed/sensitized followed by nose-only MDI-aerosol challenged murine model.

(A) Experimental timelines showing MDI exposed time points, routes. Expression levels of candidate circulating (B) mmu-miR-183-5p, (C) mmu-miR-206-3p, and (D) mmu-miR-381-3p were compared to control mice without dermal and airway MDI-aerosol exposure control (N=3; bars, s.e.m.). MDI: 4,4'-methylene diphenyl diisocyanate; ACN: acetone. (*** $P<0.001$).

Table 1.

Top 20 candidate circulating miRs identified by miRCURY™ miRs qPCR Profiling Service.

Microarray data shows differently expressed circulating miRs from dermal MDI exposure compare to vehicle control. The 20 most differentially expressed serum miR fold-changes are shown from MDI-dermal exposed mice compared to acetone (ACN) control (CTL). Microarray analysis for miR was performed with RNA extracted after 4 days following 1% MDI/ACN exposure to MDI or ACN skin painted mice.

microRNAs	Mature microRNA sequences	Human homologues	Fold changes compare to CTL
mmu-miR-1983	CUCACCUGGAGCAUGUUUUCU	N/A	−12
mmu-miR-30d-3p	CUUUCAGUCAGAUGUUUGCUGC	hsa-miR-30d-3p	−11
mmu-miR-381-3p	UAUACAAGGGCAAGCUCUCUGU	hsa-miR-381-3p	−8.5
mmu-miR-667-3p	UGACACCUGCCACCCAGCCCAAG	N/A	−8
mmu-miR-376b-3p	AUCAUAGAGGAACAUCCACUU	N/A	−7.8
mmu-miR-744-3p	CUGUUGCCACUAACCUCAACCU	hsa-miR-744-3p	−7.6
mmu-miR-206-3p	UGGAAUGUAAGGAAGUGUGUGG	hsa-miR-206	−7.1
mmu-miR-92b-3p	UAUUGCACUCGUCCCGGCCUCC	N/A	−6.3
mmu-miR-672-5p	UGAGGUUGGUGUACUGUGUGUGA	N/A	−5.8
mmu-miR-127-3p	UCGGAUCCGUCUGAGCUUGGCU	hsa-miR-127-3p	−5.4
mmu-miR-181a-1-3p	ACCAUCGACCGUUGAUUGUACC	hsa-miR-213	−4.6
mmu-miR-300-3p	UAUGCAAGGGCAAGCUCUCUUC	N/A	−4.5
mmu-miR-192-3p	CUGCCAAUUCCAUAGGUCACAG	hsa-miR-192-3p	4.5
mmu-miR-202-5p	UUCCUAUGCAUAUACUUCUUU	N/A	4.5
mmu-miR-201-5p	UACUCAGUAAGGCAUUGUUCUU	N/A	4.6
mmu-miR-153-3p	UUGCAUAGUCACAAAAGUGAUC	hsa-miR-153-3p	4.8
mmu-miR-351-5p	UCCCUGAGGAGCCCUUUGAGCCUG	N/A	5.5
mmu-miR-433-3p	AUCAUGAUGGGCUCCUCGGUGU	hsa-miR-433-3p	9.3
mmu-miR-183-5p	UAUGGCACUGGUAGAAUUCACU	hsa-miR-183-5p	11
mmu-miR-1195	UGAGUUCGAGGCCAGCCUGCUCA	N/A	13

Table 2.

Pathways enriched in potential hsa-miR-183-5p targets.

Databases	Pathways[a]	Pathway ID	Genes[b]	Target[c]	Empirical *P-value*
REACTOME	Neuronal system	REACT_13685	289	20	0.0010
REACTOME	Opioid signaling	REACT_15295	80	9	0.0012
KEGG	Gastric acid secretion	4971	74	8	0.0012
REACTOME	Platelet homeostasis	REACT_23876	81	8	0.0012
REACTOME	Potassium channels	REACT_75908	99	9	0.0013
REACTOME	Activation of kainate receptors upon glutamate binding	REACT_21312	30	5	0.0019
REACTOME	G-protein beta gamma signaling	REACT_19388	28	4	0.0020
REACTOME	Glucagon-type ligand receptors	REACT_18377	33	4	0.0025
REACTOME	Thrombin signaling through proteinase activated receptors (PARS)	REACT_21384	32	5	0.0027
REACTOME	ADP signaling through P2Y purinoceptor 1	REACT_19140	25	5	0.0030
REACTOME	**Formation of HIV-1 elongation complex containing HIV-1 Tat**	REACT_6346	42	4	0.0034
REACTOME	**HIV-1 transcription elongation**	REACT_6274	42	4	0.0034
REACTOME	**Tat-mediated elongation of the HIV-1 transcript**	REACT_6162	42	4	0.0034
REACTOME	Transmission across chemical synapses	REACT_13477	190	15	0.0052
REACTOME	Glucagon signaling in metabolic regulation	REACT_1665	33	5	0.0053
REACTOME	Inwardly rectifying K^+ channels	REACT_75918	31	5	0.0059
REACTOME	Activation of G protein gated potassium channels	REACT_75831	25	4	0.0064
REACTOME	G protein gated potassium channels	REACT_75780	25	4	0.0064
REACTOME	Inhibition of voltage gated Ca^{2+} channels via G beta gamma γ subunits	REACT_25004	25	4	0.0064
REACTOME	Signal amplification	REACT_20524	31	5	0.0066
REACTOME	G beta gamma signaling through PI3Kgamma	REACT_19290	25	4	0.0074
REACTOME	G alpha (z) signaling events	REACT_19333	45	6	0.0075
REACTOME	**Formation of HIV-1 elongation complex in the absence of HIV-1 Tat**	REACT_22201	42	4	0.0076
REACTOME	Formation of RNA Pol II elongation complex	REACT_1845	42	4	0.0076
REACTOME	RNA polymerase II transcription elongation	REACT_833	42	4	0.0076
REACTOME	Neurotransmitter receptor binding and downstream transmission in the postsynaptic cell	REACT_15370	136	10	0.0077
REACTOME	Integration of energy metabolism	REACT_1505	125	10	0.0101
REACTOME	G-protein activation	REACT_15457	28	4	0.0115
REACTOME	Transcription-coupled NER (TC-NER)	REACT_1628	44	3	0.0117
REACTOME	mRNA capping	REACT_1470	28	3	0.0118
REACTOME	RNA Pol II CTD phosphorylation and interaction with CE	REACT_6237	26	3	0.0118
REACTOME	RNA Pol II CTD phosphorylation and interaction with CE	REACT_975	26	3	0.0118
REACTOME	Regulation of insulin secretion	REACT_18325	98	8	0.0126
REACTOME	G alpha (q) signaling events	REACT_18283	186	8	0.0134

Databases	Pathways[a]	Pathway ID	Genes[b]	Target[c]	Empirical *P-value*
REACTOME	RNA polymerase II pre-transcription events	REACT_22107	58	4	0.0137
REACTOME	**Pausing and recovery of TAT-mediated HIV-1 elongation**	REACT_6143	31	3	0.0146
REACTOME	TAT-mediated HIV-1 elongation arrest and recovery	REACT_6344	31	3	0.0146
REACTOME	Aquaporin-mediated transport	REACT_23887	47	5	0.0148
REACTOME	Regulation of water balance by renal aquaporins	REACT_24023	40	5	0.0151
REACTOME	Dual incision reaction in TC-NER	REACT_2222	28	3	0.0153
REACTOME	Formation of transcription-coupled NER (TC-NER) repair complex	REACT_1941	28	3	0.0153
REACTOME	Regulation of insulin secretion by glucagon-like peptide-1	REACT_18274	43	5	0.0169
REACTOME	DNA repair	REACT_216	108	5	0.0177
REACTOME	Inhibition of insulin secretion by adrenaline noradrenaline	REACT_18339	29	4	0.0177
REACTOME	Nucleotide excision repair	REACT_1826	49	3	0.0189
KEGG	**Leishmaniasis**	5140	72	5	0.0191
KEGG	**Prion diseases**	5020	36	4	0.0209
REACTOME	**HIV-1 elongation arrest and recovery**	REACT_6259	31	3	0.0212
REACTOME	**Pausing and recovery of HIV-1 elongation**	REACT_6244	31	3	0.0212
REACTOME	Class B 2 (Secretin family receptors)	REACT_18372	90	5	0.0212
REACTOME	Formation of the early elongation complex	REACT_846	32	3	0.0231
REACTOME	**Formation of the HIV-1 early elongation complex**	REACT_6319	32	3	0.0231
REACTOME	**Transcription of the HIV genome**	REACT_6233	61	4	0.0243
REACTOME	Interferon signaling	REACT_25229	110	5	0.0281
REACTOME	**HIV-1 transcription initiation**	REACT_6332	39	3	0.0297
REACTOME	**RNA polymerase II HIV-1 promoter escape**	REACT_6253	39	3	0.0297
REACTOME	RNA polymerase II promoter escape	REACT_2089	39	3	0.0297
REACTOME	RNA Polymerase II transcription initiation	REACT_1851	39	3	0.0297
REACTOME	RNA polymerase II transcription initiation and promoter clearance	REACT_834	39	3	0.0297
REACTOME	RNA polymerase II transcription pre-initiation and promoter opening	REACT_1655	39	3	0.0297
KEGG	**Huntington's disease**	5016	183	8	0.0309
KEGG	**Long-term depression**	4730	70	6	0.0311
KEGG	**Type I diabetes mellitus**	4940	43	4	0.0316
REACTOME	RNA polymerase II transcription	REACT_1366	101	5	0.0330
KEGG	Tight junction	4530	132	9	0.0351
KEGG	Chemokine signaling pathway	4062	189	10	0.0359
REACTOME	Degradation of beta-catenin by the destruction complex	REACT_11063	67	4	0.0360
REACTOME	Signaling by wnt	REACT_11045	67	4	0.0360
REACTOME	Metabolism of carbohydrates	REACT_474	126	5	0.0370
REACTOME	GABA receptor activation	REACT_25199	53	5	0.0388
REACTOME	G alpha (S) signaling events	REACT_19327	125	6	0.0407

Databases	Pathways[a]	Pathway ID	Genes[b]	Target[c]	Empirical *P-value*
REACTOME	Interferon gamma signaling	REACT_25078	73	4	0.0444
REACTOME	Costimulation by the CD28 family	REACT_19344	77	6	0.0464
REACTOME	NFκB and MAP KINASES activation mediated by TLR4 signaling repertoire	REACT_25281	71	6	0.0473
REACTOME	Mitotic prometaphase	REACT_682	92	5	0.0477
REACTOME	Activation of GABA B receptors	REACT_25330	38	4	0.0488
REACTOME	GABA B receptor activation	REACT_25031	38	4	0.0488
REACTOME	M phase	REACT_910	96	5	0.0499
REACTOME	TRAF6 Mediated induction of proinflammatory cytokines	REACT_6782	68	6	0.0499

a. Human diseases relative pathways are shown in bold font

b. indicates the total number of genes involve in the given pathway

c. indicates the numbers of potential hsa-miR-183-5p targets in the pathways.

Table 3.

Pathways enriched in both hsa-miRs-206 and -381-3p cotargets.

Databases	Pathways[a]	Pathway ID	Genes[b]	Target[c]	Empirical *P-value*
REACTOME	Hemostasis	REACT_604	467	36	0.0003
KEGG	Adherens junction	4520	73	11	0.0004
REACTOME	Platelet activation signaling and aggregation	REACT_798	205	19	0.0007
BIOCARTA	Biocarta EDG1 pathway		27	5	0.0032
KEGG	Bacterial invasion of epithelial cells	5100	70	9	0.0041
KEGG	**Fc gamma R-mediated phagocytosis**	4666	94	9	0.0084
BIOCARTA	**Biocarta NFAT pathway**		54	6	0.0114
KEGG	**Chemokine signaling pathway**	4062	189	13	0.0130
KEGG	Regulation of actin cytoskeleton	4810	213	16	0.0131
KEGG	Shigellosis	5131	61	7	0.0149
REACTOME	Neuronal system	REACT_13685	289	17	0.0160
REACTOME	Platelet degranulation	REACT_318	78	7	0.0160
BIOCARTA	**Biocarta FMLP pathway**		37	5	0.0177
REACTOME	Response to elevated platelet cytosolic Ca^{2+}	REACT_1280	83	7	0.0184
KEGG	Vibrio cholera infection	5110	54	5	0.0192
KEGG	Pentose phosphate pathway	30	26	4	0.0196
REACTOME	Signaling by Robo receptor	REACT_19351	32	5	0.0197
REACTOME	Platelet homeostasis	REACT_23876	81	8	0.0201
BIOCARTA	Biocarta MPR pathway		34	5	0.0211
REACTOME	Sphingolipid metabolism	REACT_19323	32	4	0.0211
REACTOME	Metabolism of carbohydrates	REACT_474	126	9	0.0223
KEGG	Epithelial cells signaling in helicobacter pylori infection	5120	68	7	0.0327
BIOCARTA	Biocarta MET pathway		37	6	0.0328
BIOCARTA	Biocarta keratinocyte pathway		46	6	0.0337
KEGG	Spliceosome	3040	127	9	0.0347
KEGG	**Leukocyte transendothelial migration**	4670	116	8	0.0353
REACTOME	Formation and maturation of mRNA transcript	REACT_2039	185	12	0.0354
BIOCARTA	Biocarta PPARA pathway		58	6	0.0367
REACTOME	Signaling by PDGF	REACT_16888	122	11	0.0369
REACTOME	Downstream signal transduction	REACT_17025	93	8	0.0379
KEGG	Vascular smooth muscle contraction	4270	126	8	0.0381
REACTOME	G alpha (q) signaling events	REACT_18283	186	9	0.0393
REACTOME	Transmission across chemical synapses	REACT_13477	190	11	0.0399
KEGG	Focal adhesion	4510	199	16	0.0420
BIOCARTA	Biocarta VEGF pathway		29	5	0.0457
KEGG	RNA degradation	3018	57	4	0.0457

Databases	Pathways[a]	Pathway ID	Genes[b]	Target[c]	Empirical *P-value*
BIOCARTA	Biocarta CREB pathway		27	4	0.0482
REACTOME	Signaling to erks	REACT_12058	35	4	0.0495

[a] immune system relative pathways are shown in bold font

[b] indicates the total number of genes involve in the given pathway

[c] indicates the numbers of hsa-miRs-206 and -381-3p cotargets in the pathways.