Circular RNA *hsa_circ_0008726* Targets the *hsa-miR-206-3p*/KLF4 Axis to Modulate 4,4'-Methylene Diphenyl Diisocyanate-Glutathione Conjugate-Induced Chemokine Transcription in Macrophages

Introduction

Occupational exposure to 4,4'-methylene diphenyl diisocyanate (MDI), the most widely used monomeric diisocyanate (dNCO), is associated with occupational asthma (OA) development. Recruitment of immune cells to the lung microenvironment via secreted chemokines by alveolar macrophages may plays important roles during asthma pathogenesis. Our prior studies identified MDI/MDI-GSH-exposure downregulates endogenous human/murine(*hsa/mmu*)-microRNA(*miR*)-*206-3p*, resulting in the activation of *mmu/hsa-miR-206-3p*-regulated signaling including KLF4-mediated signaling in MØs. The *hsa-miR-206-3p*-regulated signaling activation leading to induction of chemokines and chemotaxis activities of immune cells. However, the underlying molecular mechanism(s) by which MDI/ MDI in the form of MDI-GSH conjugate exposure downregulated endogenous *hsa-miR-206-3p* expression is unclear. Circular RNAs (circRNAs) play important roles in many different biological processes by targeting endogenous miRs, affecting protein translation and gene transcription in different cell types. The circRNA expression can be regulated via outside stimuli exposures; however, whether MDI-exposure influence circRNAs expression is unknown. Several circRNAs have been identified to regulate *hsa-miR-206-3p* levels through miR binding/targeting. We hypothesize that MDI-exposure induces endogenous circRNA(s) to regulate *hsa-miR-206-3p* in MØs. The first aim of this study was to identified candidate *hsa-miR-206-3p*-binding circRNA(s) that can be regulated by MDI/MDI-GSH regulated. *The second aim of this study was to examine whether MDI/MDI-GSH regulated hsa-miR-206-3p*-binding *circRNA(s)* can indeed regulate the endogenous *hsa-miR-206-3p* in MØs. After identified the roles of endogenous circRNA(s) in regulation of endogenous *hsa-miR-206-3p* after MDI/MDI-GSH-exposure, we investigated the roles of circRNAs in regulation of *hsa-miR-206-3p*-mediated M2 macrophage-associated markers and chemokines' expressions in relation to the exposure to MDI.

Methods Collection

1. Cell culture and cell differentiation
    - THP-1, Jurkat T-cells clone E6-1, and Clone 15 HL-60 cells were obtained from ATCC.
    - Enhanced differentiated THP-1 macrophages were prepared using media containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce differentiation for 3 days and then enhanced by refeeding fresh media after removing PMA containing media for additional 3 days.
    - Differentiated eosinophils were prepared by culturing Clone 15 HL-60 cells in RPMI-1640 media containing 0.5mM butyric acid for 7 days.
2. MDI-GSH conjugation
    - 10 mM GSH solution was prepared in 200 mM sodium phosphate buffer (pH= 7.4).
    - MDI-GSH conjugation was prepared by adding MDI/acetone directly into GSH solution. End-to-end mixing for 1 h at 25 °C.
3. Transient transfections of *hsa_circ_0008726* siRNAs, miR-mimics/inhibitors and *hsa_circ_0008726* overexpression
    - Plasmid DNAs were transfected into THP-1 macrophages using Mirus *Trans*IT-2020 transfection reagent according to manufacturer's instructions.
    - miR-mimics/inhibitors or *hsa_circ_0008726* siRNAs were transfected into THP-1

macrophages using Lipofectamine RNAiMAX transfection reagent according to manufacturer's instructions.

4. M2 macrophage-associated transcription factors, markers, and chemokines expression (transcripts and proteins)
    - Total RNA was isolated using *mirVana*™ miR isolation kit according to manufacturer's instructions.
    - TaqMan gene expression assays for *KLF4*, *CD206, TGM2, CCL17, CCL22* and *CCL24* were obtained from ThermoFisher Scientific.
    - Real-time PCR assays were performed on Applied Biosystems 7500 RT-PCR System.
    - Secreted M2 macrophage-associated chemokines CCL17, CCL22, and CCL24 proteins in cell-free conditioned media were determined by ELISA. The human CCL17, CCL22, and CCL24 ELISA kits were obtained from R&D systems. ELISA were performed according to manufacturer's instructions.
5. Validation of miR targets by Argonaute (AGO) immunoprecipitation
    - Immunoprecipitation (IP) of the miR-containing RNA inducing silencing complex (miR/RISC) and miR targeting mRNAs was performed using the miRNA target IP kit (Active Motif) according to manufacturer's instructions.
    - RNA was isolated from Immunoprecipitated miR/RISC/circRNA(s) complexes using *mirVana*™ miR isolation kit according to manufacturer's instructions.
    - RT-qPCR analysis of *hsa_circ_0008726* were performed on RNA isolated from Immunoprecipitated miR/RISC/circRNA(s) complexes.
    - Real-time PCR assays were performed on Applied Biosystems 7500 RT-PCR System.
6. Chemotaxis assays and quantification of migrated cells
    - 3 μm pore size Transwell™ assay insert was used for chemotaxis/cell migration assays.
    - Either Jurkat T-cells or differentiated Clone 15 HL-60 cells were added into Transwell™ assay inserts and allowed those cells responded to conditioned media obtained from THP-1 macrophages transfected with *hsa_circ_0008726* overexpression plasmid.
    - Migrated immune cells were quantified by using CyQUANT® GR proliferation assay according to manufacturer's instructions.

Citations – Publications based on the dataset.

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