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Alteration of MicroRNAs Targeted Integrins by PD-MSCs Transplantation Is Involved in Hepatic Regeneration in a Rat Model with BDL

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Placenta-derived mesenchymal stem cells (PD-MSCs) are promising candidates for cell-based therapy in regenerative medicine. The migration and homing potential of PD-MSCs to injured sites is a critical property of MSC engraftment. MicroRNAs (miRNAs) have recently been shown to regulate the critical functions of MSCs, such as proliferation, survival, and migration. The objective of the present study was to identify the miRNA and target genes involved in PD-MSCs homing in a bile duct ligation (BDL) rat model. We selected candidate miRNAs targeting genes for PD-MSCs homing based on microarray analysis. PD-MSC engraftment in BDL-injured rat liver was identified by immunofluorescence assay and human-specific Alu gene expression by quantitative real-time polymerase chain reaction (qRT-PCR) one week after transplantation. Compared with migrated naïve PD-MSCs under hypoxic and normoxic conditions (Hyp/Nor), the transplanted group with PD-MSCs (Tx) showed distinct differences in miRNA expressions in BDL-injured rat liver. We also validated the miRNAs and their target genes for PD-MSCs homing. The expressions of integrin α4 (ITGA4) and integrin α5 (ITGA5) target genes for miR-199a-5p and miR-148a-3p were significantly upregulated in the Tx group ($p<0.05$). In addition, integrin β1 (ITGB1) and integrin β8 (ITGB8) were upregulated by suppressing miR-183-5p and miR-145-5p, respectively. These results demonstrated that PD-MSCs regulate miRNA expression related to the integrin family for their homing effects on the BDL-injured rat liver. The findings further suggest that miRNA-mediated regulation of the integrin family contributes to the therapeutic efficacy of PD-MSCs in the rat hepatic fibrosis model by BDL.

Key words : Integrins, liver failure, microRNA, Placenta-derived mesenchymal stem cells (PD-MSCs), stem cell migration

Introduction

Hepatic failure induced by chronic liver injury is a common response to liver diseases that are difficult to cure with significant morbidity and mortality worldwide [30]. Despite the powerful regenerative ability of the liver, repetitive and continuous hepatic damages lead to progressive liver fibrosis and ultimately end-stage liver disease [40]. Although a number of specific therapies for liver fibrosis have been developed, liver transplantation is the most effective treatment for patients with chronic liver disease. However, this treatment has many limitations such as organ shortages, immunological rejection and high medical costs [4, 18].

Recently, multipotent mesenchymal stem cells (MSCs) have been increasingly applied in clinical trials for the treatment of various diseases, especially liver disorders based on their unique therapeutic properties in preclinical and clinical studies [www.clinicaltrials.gov, 7, 35]. MSCs are able to self-renewal and differentiation into multiple cell lineages, including hepatocytes, which play key roles in tissue healing and regenerative medicine [1, 27]. MSCs are found in almost tissues, including bone marrow, adipose tissue, placenta and cord blood as well as other tissues [3]. Among them, placenta-derived MSCs (PD-MSCs) obtained from fetal tissue origin have advantages for strong immunomodulatory capacity, anti-inflammatory effects and easily accessible to obtain abundant cells in vitro with free from ethical concern [20]. These properties are considered to maximize the therapeutic efficacy of PD-MSCs-based therapy in regenerative medicine. Furthermore, PD-MSCs can migrate toward injured areas and secrete various molecules to create a microenvironment in response to cellular damage signals which induce homing of MSCs [31]. The homing capacity of MSCs is also affected by the dynamic expression of cell-adhesion molecules including integrins during their engraftment.

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Previous studies reported that PD-MSCs enhanced endothelial cells migration in a hypoxic condition via integrin alpha 4 (ITGA4) and Ras homolog (RHO) signaling, and integrin-dependent signaling improved MSC migration into injured target tissues [8].

MicroRNAs (miRNA) are small, non-coding RNAs (~22 nucleotides) that can regulate protein-coding genes by binding to their target mRNAs through various post-transcriptional mechanisms [25]. MiRNA have recently been shown to play critical functions in MSCs to regulate the cellular properties, proliferation, migration and paracrine activity [24]. MiRNAs are also involved in regulating multiple factors for liver regeneration [6, 19]. Recent reports suggest that miRNAs can control the expression levels of many important adhesion molecules [33]. The integrin family of cell matrix adhesion receptors is a key regulator of cell extracellular matrix interactions and certain miRNA involved in regulating the specific integrin. It has been reported that miR-31 and miR-92a suppressed the expression of mRNA encoding integrin α5, and miR-30 and let-7a regulated integrin β1 expression [2, 5, 32]. Furthermore, upregulation of miR-10b promoted the migration of bone marrow MSCs in vitro by targeting E-cadherin [39]. However, the profiles and functions of miRNAs induced by PD-MSCs transplantation on the MSC homing process for liver regeneration in the BDL-injured rat model remains poorly understood. Therefore, the objective of this study was to identify the expression of miRNAs and their target genes associated with cell-adhesion molecules for PD-MSCs homing and to confirm the correlation between miRNAs and their predicted target genes in a BDL-rat model transplanted with PD-MSCs.

Materials and Methods

Cell culture

The collection of placental samples and their use for research purposes were approved by the Institutional Review Board (IRB) of CHA Gangnam Medical Center, Seoul, Korea (IRB07-18). PD-MSCs were harvested as described previously [22] and cultured in α-modified minimal essential medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin (Gibco), 1 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA) and 25 ng/ml human fibroblast growth factor-4 (PeproTech, Rocky Hill, NJ, USA). Cells were maintained below 5% CO_2 at 37℃. To induce hypoxia condition, the cells were placed in a hypoxia chamber (C-chamber, BioSpherix, Ltd., Lacona, NY, USA) and maintained at 1% O_2 and 37℃ in a 5% CO_2 humidified condition.

Animal models and transplantation of PD-MSCs

Seven-week-old male Sprague-Dawley rats (Orient Bio Inc., Seongnam, Korea) were housed under specific pathogen-free conditions and generated chronic liver cirrhosis using common BDL as previously described [19]. After one week post-surgery, PD-MSCs (2×10^6 cells, 9-10 passages) were stained with the PKH67 Fluorescent Cell Linker Kit (Sigma-Aldrich) and transplanted through the tail vein in the transplanted group (Tx ; n=20) . Non-transplanted BDL group (NTx; n=20) were maintained as the normal group (Nor; n=5). The liver tissue samples were harvested at 1, 2, 3, and 5 weeks from rats in all groups. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of CHA University, Bundang, Korea (IACUC-190048)

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from PD-MSCs and rat liver tissues using TRIzol (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from 500 ng total RNA using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. cDNA synthesis for miRNAs was performed using miR-X miRNA First-Strand Synthesis kit (Takara Bio, Kusatsu, Shiga, Japan). qRT-PCR was performed using SYBR Master Mix (Roche, Basel, Switzerland) in a CFX Connect™ Real-Time System (Bio-Rad,Hercules, CA, USA). Target gene and miRNA expression were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 small RNA, respectively. The sequences of the primers for target genes and miRNAs are shown in Table 1. All reactions were performed at least in triplicate.

Transwell migration assay

The migration of PD-MSCs was performed using a Transwell assay under normoxic and hypoxic (1% O_2) conditions. PD-MSCs (2×10^4 cells/well) were added into Transwell membrane inserts (8 μm pore size; Corning, NY, USA) and incubate for 24 hr at 37℃. The migrated cells from each condition were analyzed for miRNA expression.

Table 1. Primer sequences used in the present study for qRT-PCR analysis

Analysis	Genes	Sequences
mRNA	Rat ITGA4	F: 5′-GGAAGCCCCAGTGGAGAAC-3′ R: 5′-ATTGTCACTCCCAGCCACTGA-3′
	Rat ITGA5	F: 5′-AGCCCCAGGGACTTACAACT-3′ R: 5′-CTTCATAGGGCCCATCTTCA-3′
	Rat ITGB1	F: 5′-AACAGTGAAGACATGGATGC-3′ R: 5′-CTCTCTCTTCCTGCACACAC-3′
	Rat ITGB8	F: 5′-AGTGCCCTCCAAGCTTAACCAC-3′ R: 5′-CGTCCCACCTCTCTCTCGAA-3′
	Rat GAPDH	F: 5′-TCCCTCAAGATTGTCAGCAA-3′ R: 5′-AGATCCACAACGGATACATT-3′
miRNA	rno-199a-5p	F: 5′-CCCAGUGUUCAGACUACCUGUUC-3′
	rno-148a-3p	F: 5′-UCAGUGCACUACAGAACUUUG-3′
	rno-183-5p	F: 5′-UAUGGCACUGGUAGAAUUCACU-3′
	rno-145-5p	F: 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′

Analysis of microRNA profiling

To analyze microRNAs profiling, miRNAs sequencing experiments was performed using the Illumina platform (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The construction of small RNA libraries and sequences were performed by LAS Inc. (Gimpo, Republic of Korea). The miRNA expression levels were estimated by transcripts per 10 million reads (TPTM) according to normalizing the miRNA counts with the total number of clean reads in the small RNA libraries.

Statistical analysis

The experimental data are expressed as the means± standard deviation. Statistical analysis was performed using Student's t-test, and p-value < 0.05 was considered statistically significant. Each experiment was performed at least in triplicate.

Results

MicroRNA profiling of migrated PD-MSCs under hypoxic condition and in BDL-injured rat liver

To determine miRNA-mediated gene expression related to the PD-MSC migration and homing toward injured sites, we analyzed miRNA profiles in BDL-induced cirrhotic liver tissues at 1 week and 2 weeks after PD-MSCs transplantation and migrated PD-MSCs under hypoxic conditions using miRNA microarray (Fig. 1A). In the heatmap analysis, 54 miRNAs showing differential expression between groups were selected. In particular, the expression of miRNA in the transplanted /non-transplanted group at 1 week (Tx/NTx 1w) was significantly different compared to that in the transplanted /non-transplanted group at 2 weeks (Tx/NTx 2w). Comparative expression analysis of scatter plot between hypoxic and normoxic conditions revealed significant change in miRNA expression. Moreover, in BDL-rat liver, remarkably different event of miRNA expression between transplanted /non-transplanted group at 1 week (Tx/NTx 1w) and transplanted /non-transplanted group at 2 weeks (Tx/NTx 2w) was identified (Fig. 1B, Fig. 1C). These data suggest that variable miRNA expression regulates PD-MSCs migration under hypoxic condition and in the BDL-injured rat liver transplanted with PD-MSCs.

Engraftment of PD-MSCs in BDL-injured rat liver.

The homing of transplanted PD-MSCs to injured sites is a critical property of engraftment of MSCs [12]. After tail-vein transplantation of PD-MSCs into BDL-injured rat, PD-MSC engraftment in the rat liver was identified by immunofluorescence assay using PKH67(+) signals, and genomic human Alu expression by qRT-PCR. As shown in Fig. 2A, PKH67-positive green signals in the frozen liver sections of BDL-injured rat were found at 1 week and 2 weeks after transplantation (Fig. 2A). Also genomic human Alu expression was increased at 1 week and gradually decreased after 2 weeks (Fig. 2B). These data suggest that PD-MSCs successfully migrated to the damaged liver tissue and engrafted following their administration as a live condition for a short term, which in turn led to providing the signals through paracrine effects on damaged hepatocytes.

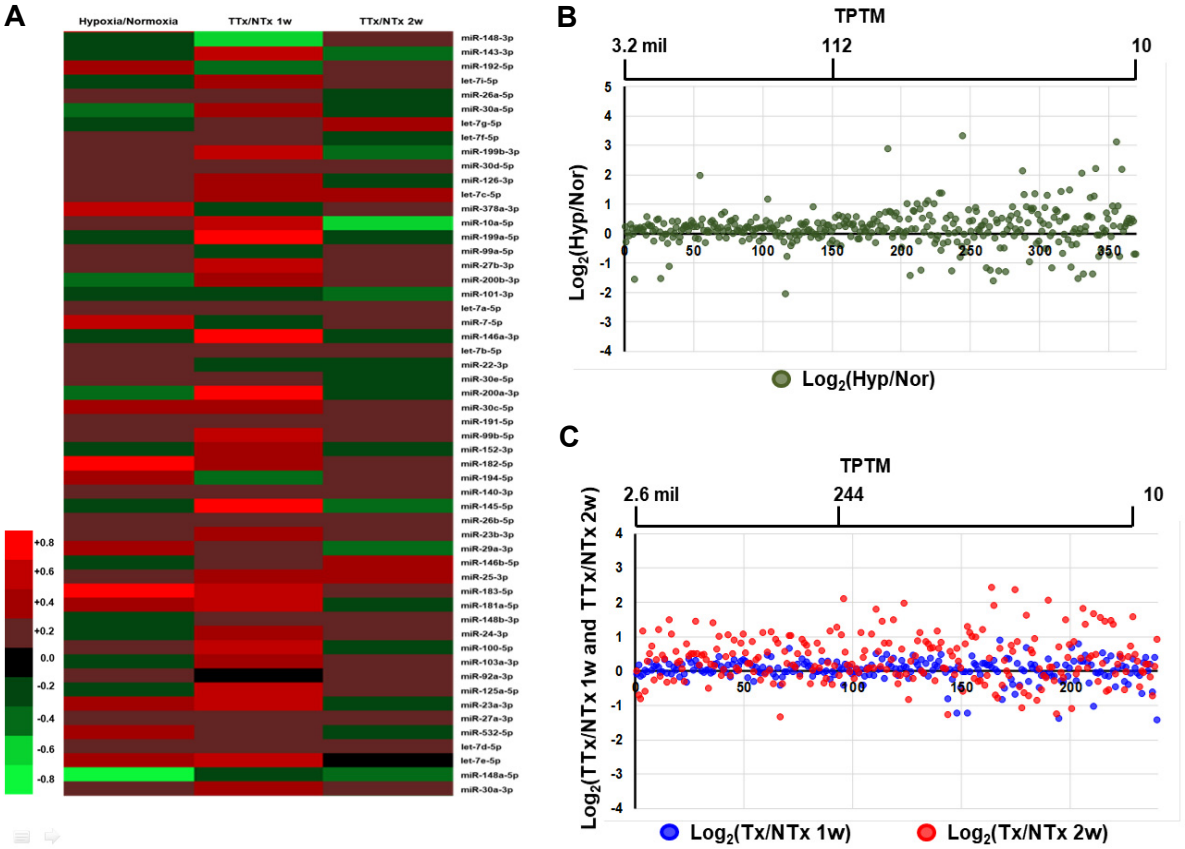

Fig. 1. MiroRNA expression profiling of migrated PD-MSCs under hypoxic condition and in BDL-injured rat liver. (A) Heatmap showing the different expression of miRNA in migrated PD-MSCs under hypoxic versus normoxic conditions, and transplanted (Tx) versus non-transplanted (NTx) groups at one week (Tx/NTx 1w) and two weeks (Tx/NTx 2w). (B) Scatterplot of miRNA in migrated PD-MSCs under hypoxic and normoxic conditions (C) Scatterplot of miRNA in Tx/NTx 1w compared to Tx/NTx 2w groups.

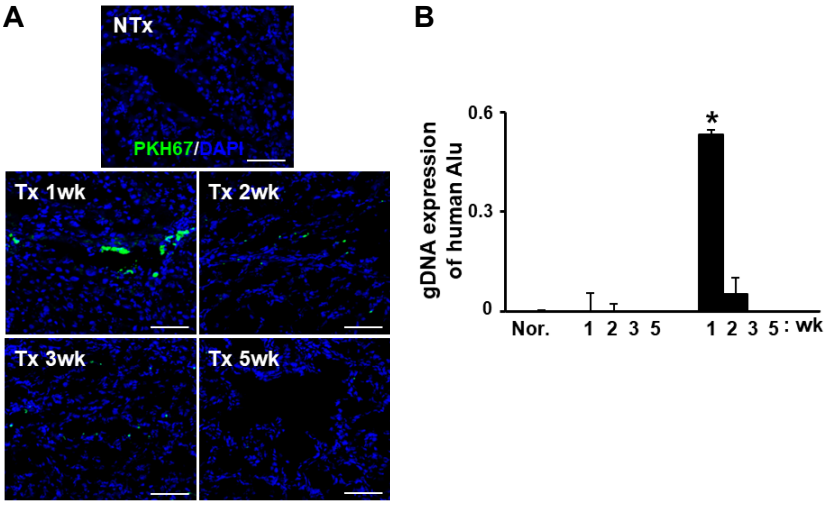

Fig. 2. PD-MSC engraftment in BDL-injured rat liver. (A) Representative images of PKH67 (green)-labeled PD-MSCs in BDL-injured rat liver by immunofluorescence microscopy at 1, 2 and 5 weeks after PD-MSCs injection. (B) Human-specific genomic Alu expression in BDL-injured rat liver by qRT-PCR * NTx vs. Tx groups.

Decreased miRNA-199a-5p and miRNA-148a-3p by PD-MSCs regulate the expression of targeting integrin α4 and α5 in BDL-injured rat liver

One of the key functions of PD-MSCs for cell-based therapies is the ability to home to the sites of damaged tissues. Integrins are known to play an important role in cell migration. Validated expression of miRNAs targeted to integrins revealed distinct differences in the expression of integrin.

From miRNAs database, we analyzed that miRNA-199a-5p and miRNA-148a-3p are predicted to ITGA4 and ITGA5, respectively. To determine the correlation between miR-199a-5p and ITGA4 in the BDL rat liver after PD-MSCs transplantation, qRT-PCR was applied to evaluate the expression levels of miR-199a-5p and ITGA4A. As shown in Fig. 1A, miR-199a-5p expression was upregulated in non-transplanted (NTx) BLD rat liver compared with normal liver tissues, and the mRNA level of predicted target gene, ITGA4, was decreased. Whereas ITGA4 expression was significantly increased in the transplanted group (Tx) compared with the NTx group at 1 week and 2 weeks (Fig. 3A, *p<0.05). Generally, it is well known that ITGA5 expression is one of major factors on adhesion molecules involved in migration of cell [17]. Moreover, the ITGA5 expression of the Tx group was remarkably upregulated compared with the NTx group by suppressing rno-miR-148a-3p (Fig. 4B, *p<0.05). Especially, their expression levels were higher (max. 50-folds) than those of ITGA4. These results suggest that decreased miR- 199a-5p and miR-148a-3p regulates the integrin family for homing and engraftment of PD-MSCs in a BDL-injured rat model as well as ITGA4 and ITGA5 might be important factors in PD-MSC homing in damaged liver tissues.

Down-regulated miRNA-183-5p and miRNA-145-5p by PD-MSCs regulate the expression of targeting integrin β1 and β8 in BDL-injured rat liver

Basically, integrin consists of two components such as integrin alpha and integrin beta subunits among several in-

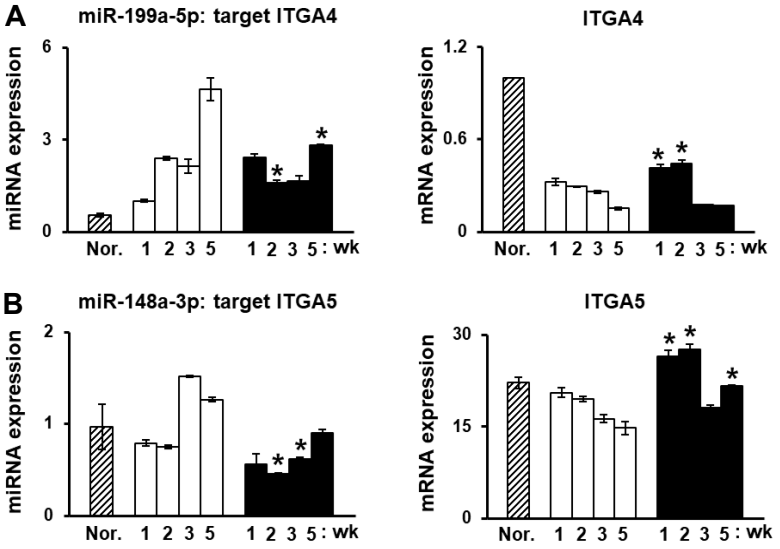

Fig. 3. MiRNAs induced by PD-MSCs regulate integrin α subunits in BDL-injured rat liver. (A) ITGA4-targeted rno-miR199a-5p expression and (B) ITGA5-targeted rno-148a-3p expression in BDL-injured rat liver at 1, 2, 3 and 5 weeks after PD-MSCs injection as determined by qRT-PCR. The experiments were performed at least in triplicate. Data from each group are indicated as the mean ± SD, determined by Student's t-test. * p<0.05 versus NTx group.

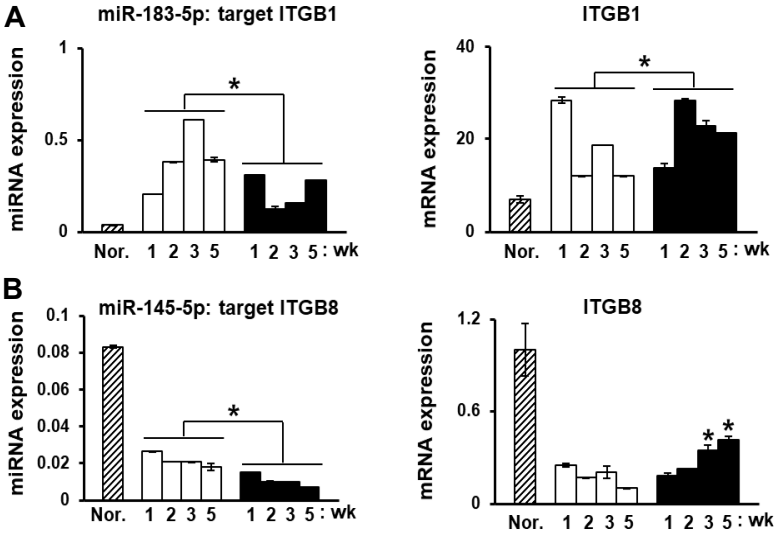

Fig. 4. MiRNAs induced by PD-MSCs regulate integrin β subunits in BDL-injured rat liver. (A) ITGB1-targeted rno-miR183-5p expression and (B) ITGB8-targeted rno-miR-145-5p expression in BDL-injured rat liver at 1, 2, 3 and 5 weeks after PD-MSCs injection as determined by qRT-PCR. The experiments were performed at least in triplicate. Data from each group are indicated as the mean ± SD, determined by Student's t-test. * p<0.05 versus NTx group.

tegrin subunits [16]. The major integrin-to-integrin complex is ITGA5 and ITGB1 in cellular events including migration and homing [34]. To validate the expression of the miRNAs, miR-183-5p and miR-145-5p, and the predicted targets of each miRNA in the BDL rat liver after PD-MSCs transplantation, the expression levels of these miRNAs and mRNAs were examined using qRT-PCR analysis. As shown in Fig. 4A, the expression of miR-183-5p was significantly downregulated and the target gene ITGB1 expression was correlatively increased in the Tx group compared to those in the NTx group (Fig. 4A, $*p<0.05$). Additionally, the expression of miR-145-5p and target gene ITGB8 revealed significant correlation in the Tx group compared to the NTx group. Comparative expression levels of target genes, ITGB1 and ITGB8, between 1 week and 5 weeks revealed significant increases by suppressing miRNAs, miR-183-5p and 145-5p, respectively. Especially, miRNA-183-5p and their target IGTB1 levels were higher than those of miRNA-145-5p and ITGB8. These results suggest that alterative miRNA expression regulates the integrin family for homing and engraftment of PD-MSCs in a BDL-injured rat model.

Discussion

Placenta derived-MSCs (PD-MSCs) are promising candidates for stem cell therapy in the treatment of various degenerative diseases. The efficacy of PD-MSCs in cell-based therapies depends on their homing ability and engraftment into the injury site [14]. Recent studies have shown that modulation of miRNA expression by MSCs has been shown to change their biological properties that drive cellular adhesion between MSCs and injured cells [9, 10]. Moreover, the potential of PD-MSCs engraftment has also been associated with regulation of miRNAs expression in a liver-injured rat model [19]. In the present study, different profiles of miRNA were confirmed in migrated PD-MSCs under hypoxic conditions and in the PD-MSCs transplanted BDL-induced cirrhotic liver tissues at 1 week and 2 weeks. In addition, the relevance of miRNAs in the PD-MSCs transplanted BDL-induced cirrhotic liver tissues (Tx group) was identified in the experiments showing that different expression profiles related to the integrin targeting miRNAs (e.g., miR-199a-5p, miR-148a-3p, miR-183-5p, and miR-145-5p).

Several cytokines secreted from injured tissues have been demonstrated to stimulate the migration of MSCs by interacting with their receptors and activating migration-related signaling cascades. MiRNA also found to affect cell migration at downstream of cytokines and receptors [15]. Especially, miR-27b and miR-27a have been reported to affect the migration of MSCs through targeting stromal cell-derived factor (SDF)-1 and its chemokine receptor type 4(CXCR4) [26]. Also, phosphatase of regulating liver-1(PRL-1)-overexpressed PD-MSCs enhanced miRNA-mediated MSC migration through integrin-dependent signaling with improvement of liver regeneration [19]. Based on these results, PD-MSCs may have multiple effects on their migration and tissue regeneration through altered expression of cell adhesion molecules affecting by miRNAs in the injured tissues.

Among the differently expressed miRNAs in the BDL-induced liver tissues (NTx), miR-199a-5p and miR-148a-3p were found to be capable of regulating the expression levels of integrin α4 (ITGA4) and integrin α5 (ITGA5), respectively. Downregulation of miR-199a-5p increased the expression of ITGA4 in the Tx group compared to those in the NTx group. MiR-199a-5p has been demonstrated to participate in the regulation of multiple biological processes, including cell proliferation, migration and invasion [36, 38]. It was shown that miR-199a-5p protected hepatocyte damage induced by bile acid [11] and functioned as a tumor suppressor by downregulating ITGA3 [29]. Also, ITGA4 involved in increase MSC homing to the bone marrow [21]. Comparison with these data, our results indicate that PD-MSCs play an important role in promoting their migration by regulating miR-199a-5p expression for ITGA4 in BDL-induced rat liver tissues. The expression levels of integrin α5 (ITGA5) were also significantly increased by downregulation of miR-148a-3p in the Tx group. Especially, their expression levels were higher than those of ITGA4. ITGA5 was found to be regulated by several miRNAs (e.g., miR-31, miR-17-92 cluster, and miR-148b) [5]. Wu *et al* [37] reported that overexpression of miR-143a-3p reduced laryngeal squamous cell carcinoma cell migration and proliferation.

Integrins are αβ heterodimeric cell-surface receptors that facilitate cell adhesion and migration [16]. Among the integrin superfamily, α5β1 is one of the best characterized integrins for cell migration [34]. Like most integrins, integrin β1 (ITGB1) is also regulated by multiple miRNAs. The miRNA reported to directly regulate ITGB1 expression include miR-29b and miR-124a [13, 28]. Chen *et al* [5] reported that ITGB1 is a critical factor for cell adhesion and invasiveness affected by miR-183, which led to negative regulation on the invasive activity of endometrial stromal cells. It has

been reported that integrin α5β1 mediates MSC migration at sites of vascular remodeling by activation of focal adhesion kinase and platelet-derived growth factor receptor-β phosphorylation [34]. Our results showed that PD-MSCs induced significantly downregulated miR-183-5p expression resulting in upregulation of ITGB1 target gene expression in the BDL-injured rat liver. These data suggest that PD-MSCs in vivo contribute to the enhanced cell migration regulated by miR-183-5p and target gene ITGB1 expression. In addition, the expression of miR-145-5p by PD-MSCs was markedly downregulated during the experimental period, and its target gene, ITGB8, was gradually increased in the BDL-injured rat liver. It has been reported that miR-145 was identified as a tumor suppressor in a variety of cancers, and downregulated the expression of ITGB8 in human corneal epithelial cells [23].

The therapeutic effect of MSCs depends on their ability to home the injured site, which is affected by several factors derived from MSCs as their paracrine actions. Many studies have demonstrated that miRNAs may be involved in the paracrine action of MSCs [14, 15]. We confirmed that PD-MSCs successfully migrated to the damaged rat liver tissue and engrafted for 2 weeks after intravenous injection. During the 5-week experimental periods, PD-MSCs-transplantation in a liver failure rat model induced alterative expression of miRNA targeting integrin genes, which lead to better migration and engraftment of PD-MSCs into injured liver tissues, and enhancement of liver regeneration. Among the various mechanisms regulating integrin expression, miRNAs regulated by PD-MSCs have an important target in liver regeneration. Further study is ongoing to miRNA target binding validation by luciferase reporter assay and requires the elucidation of detailed mechanism..

In conclusion, the present study demonstrates that PD-MSCs regulate alterative miRNA expression related to integrin family for their homing effects on BDL-injured rat liver. These findings provide a fundamental mechanism for miRNA-mediated effects of PD-MSCs transplantation in hepatic regeneration and support the development of stem cell-based therapy.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 담관결찰 쥐 모델에서 태반유래중간엽줄기세포 이식에 의한 miRNA 표적 인테그린 변화의 간재생 효과

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태반유래 중간엽줄기세포(PD-MSCs)는 재생의학에서 세포기반치료제로 잘 알려진 세포군이다. PD-MSCs의 손상된 부위로의 이동과 호밍 기능은 MSC 생착의 중요한 특성이다. miRNA는 최근 MSC의 증식, 생존 이동과 같은 중요한 기능을 조절하는 것으로 알려져 있다. 본 연구의 목적은 담관결찰(BDL) 쥐 모델에서 PD-MSCs 호밍에 관련된 miRNA 및 표적 유전자를 동정하는 것으로, 마이크로어레이 분석을 이용하여 PD-MSCs 호밍에 관여하는 유전자 표적 miRNA를 선별하였다. BDL 쥐모델에 PD-MSCs을 이식한 일주일 후 간 조직에서 PD-MSCs 생착여부는 면역형광분석법과 qRT-PCR에 의한 인간 Alu유전자 발현으로 확인되었다. 저산소 및 정상조건(Hyp/Nor)에서 이동한 PD-MSC에 비하여, PD-MSCs 이식한 BDL군 간 조직에서 miRNAs 발현의 차이가 크게 나타났으며, PD-MSCs 호밍 관련 miRNA와 표적유전자를 검증하였다. miR199a-5p 및 miR-148a-3p에 대한 표적 유전자 인테그린 α4 (ITGA4)와 α5 (ITGA5)의 발현은 이식(Tx)그룹에서(p<0.05) 유의하게 상향 조절되었다. 또한 인테그린 β1 (ITGB1)과 β8 (ITGB8)의 발현은 miR-183-5p 및 miR-145-5p억제에 의하여 크게 증가되었다. 따라서 이러한 결과는 BDL에 의해 손상된 쥐간에서 PD-MSCs가 호밍효과을 위해 인테그린 그룹과 관련된 miRNA 발현 조절에 관여함을 나타내었다. 본 연구결과는 miRNA에 의한 인테그린 그룹 조절기능이 BDL에 의해 유도된 간섬유증 쥐모델에서 PD-MSCs의 치료효과에 기여할 수 있음을 시사한다.