Original Research Article

Tracking of intraperitoneally and direct intrahepatic administered mesenchymal stem cells expressing miR-146a-5p in mice hepatic tissue

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ABSTRACT

Given the functional drawbacks in the efficacy and safety of conventional miRNA delivery approaches, we aimed to assess the fate of mesenchymal stem cells (MSCs) expressing miR-146a-5p into the mice hepatic tissue. MSCs were xeno-transplanted through direct intrahepatic and intraperitoneal routes into immunocompetent and cyclosporine A treated BALB/c mice. DNA extracts from murine organs 24 h and 28 days after xenotransplantation were analyzed by PCR method for detection of human-specific Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) sequence. Xeno-transplanted naive MSCs could not be detected after 24 h and 28 days post-infusion at the various organs of mice. Considering the miR-146a-5p expression in MSCs together with negative results of cell engraftment in hepatic tissue, the present study suggests the cell-free secretome-based miR-146a-5p delivery for modulation of altered miRNA expression in hepatic stellate cells.

***Keywords*:** MicroRNA, intrahepatic, intraperitoneal, xenograft, tracking

INTRODUCTION

Liver fibrosis is a common feature of all chronic liver diseases and is defined by persistent and excessive production of extracellular matrix proteins by activated Hepatic Stellate Cells (HSC) known as myofibroblasts [1]. If liver fibrosis is uncured, it can disturb the normal hepatocellular function and liver architecture that lead to cirrhosis and liver failure [2,3]. Therefore, the majority of studies have focused on the development of anti-fibrotic treatments that can suppress the proliferation and activation of hepatic stellate cells for the amelioration of chronic liver diseases [4]. MicroRNAs (miRNAs) or small non-coding RNAs are a subset of regulatory non-coding RNAs that repress target gene expression by binding to the 3′ untranslated regions (3′UTR) of target miRNA [5] resulting in the suppression of translation or the progression of mRNA decay [6]. Various studies have demonstrated that miRNAs have pivotal roles in diverse physiological and pathological processes [7] such as tissue regeneration, cellular proliferation, differentiation [8] and initiation of many diseases [9]. In a recent decade, the role of miRNAs in the trans-differentiation of quiescent HSCs to matrix-producing myofibroblasts and the development of liver fibrosis being investigated. Results of previous studies had confirmed the effect of downregulated expression of miR-146a-5p on HSCs activation and trans-differentiation to myofibroblasts through signaling pathways in the liver [10]. Accumulating evidence has shown that overexpression of mir-146a-5p in the activated HSCs could have therapeutic potential in the amelioration of liver fibrosis [10] indicating that tuning miRNAs expression profile maybe act as a promising method for the promotion of novel therapeutic approaches [11]. In spite of beneficial and therapeutic potential of miRNAs, *in vivo* delivery of these naked nucleotides to the specific organs including fibrotic liver is hindered by issues including, immunogenicity, short half-life, low uptake by target cells and instability in the body fluid due to rapid degradation by nucleases [12-14]. For overcoming these obstacles, attempts should be made to promote effective miRNAs delivery systems that are able to allow long term expression of miRNAs in both in vitro and *in vivo* conditions [13]. In recent years, a number of continuous efforts to specifically deliver therapeutic miRNA to diseased organs were progressing by using various technique including both viral and nonviral

approaches [15]. Considering the drawbacks in the efficacy and safety of conventional miRNA delivery approaches, cell-based miRNA therapy could be a suitable alternative to the current viral and non-viral delivery systems [15]. Previous studies had reported that Mesenchymal Stem Cells (MSCs) could alleviate tissue damage or regeneration by secreting various biological factors [16]. Additionally, previous reports revealed beneficial biological effects of MSCs on tissue protection or regeneration mediated by transferring MSCs derived miRNAs to the target organ. Indeed, MSCs derived miRNAs could modulate compromised miRNA expression profile in damaged cells [16]. Considering the involvement of miR-146-5P down-regulation in the pathophysiology of liver fibrosis, MSCs derived miR-146-5P transfer to the hepatic tissue due to therapeutic and homing properties of these cells [17] could offer unique expression in the damaged tissue and promote the efficacy of miRNA therapy. In spite of promising reports from different studies regarding the beneficial effects of mesenchymal stem cells in treating various disorders including liver fibrosis, there are many controversies between results from larger-scale clinical trials [17,18]. Thus to optimally explore the therapeutic potential of human placenta-derived MSCs in treating liver fibrosis, it is essential to identify the miR-146a-5p expression level in these cells. Also, for successful stem cell-based gene therapy and translation to clinical trials, it is prerequisite to achieve comprehensive knowledge about the effect of multiple parameters including cell delivery routes that impact the viability, biodistribution pattern, engraftment and fate of transplanted cells in the living systems [19]. In this study, following analysis of miR-146a-5p expression profile in HP-MSCs, we decided to investigate the biodistribution and engraftment of unmodified human MSCs through the peritoneal cavity and directly into the liver parenchyma of the immunocompetent and cyclosporine A treated BALB/c mice. We consciously used the healthy xenograft mice model, because results of the previous studies have been revealed that hepatic inflammation or injury could not affect the homing characteristics of the transplanted cells into the liver parenchyma [20,21]. Also, for better understanding of the host immune reaction and immunomodulatory role of MSCs in the xenograft models, we investigated the fate of naive human MSCs after administration into the immunocompetent mice.

MATERIALS AND METHODS

Isolation and culture of mesenchymal stem cells (MSCs) from human placenta

The placentas were obtained in sterile conditions from healthy pregnant mothers after caesarean sections following full informed and written consent in accordance with the guidelines of the ethics committee of Tehran University of Medical Sciences (TUMS) (registration number: IR.TUMS.VCR.REC.1397.010). Blood samples of all donors were examined for transmissible infectious agents including Human Immunodeficiency Virus-1 (HIV-1), hepatitis B, hepatitis C, and cytomegalovirus. The amniotic and chorionic membranes of placenta were mechanically removed. Placenta from fetal side was picked, rinsed, and cut into small pieces approximately 1 mm^3. The tissue fragments were washed intensively three times with 9 % sodium chloride solution to remove the remaining blood, before being incubated with 0.1 % collagenase IV (Sigma, St. Louis, MO, USA) for 3 h at 37 ˚C, with manually shaking every 30 min. Then, 9 % sodium chloride solution was added and the mixture was shaken and centrifuged at 2000 rpm for 3 min. The supernatant was discarded and the pellet was suspended in complete MSCs culture medium containing Dulbecco's Modified Eagle's Medium (DMEM, Bioidea, Tehran, Iran) supplemented with 10 % Fetal Bovine Serum (FBS; Gibco, Grand Island, NY, USA) and 2 % penicillin/streptomycin (Gibco, Grand Island, NY, USA). Primary cultures were maintained in a 37 ˚C humidified 5 % CO_2 incubator. The non-adherent cultured cells were removed in 2 days. Following colony formation, MSCs were singled using enzymatic digestion (Trypsin-EDTA solution 0.25 %, Sigma, St. Louis, MO, USA) and sub-cultured for characterization and following experiments.

Cell surface antigen characterization

HP-MSCs were isolated and suspended in 3 % bovine serum albumin (Sigma Aldrich) in phosphate-buffered saline (Sigma, St. Louis, MO). Cells were incubated for 1 h at 4 ˚C with the specific antibodies or isotype control antibodies (IgG2a, from Santa Cruz Biotechnology). The immunophenotype of HP-MSCs after 3 passage was assessed by monoclonal antibodies against the human cell surface markers CD105, CD73, CD90, CD34, CD44 and CD45 (eBioscience, San Diego, CA, USA) and analyzed with a flow cytometer.

Multilineage differentiation of HP-MSCs

Adipogenic differentiation of HP-MSCs was assessed by culturing cells in the induction medium containing DMEM supplemented with 10 % FBS, 250 nmol/L dexamethasone and 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO, USA). Osteogenic differentiation of HP-MSCs was assessed by culturing cells in an induction medium containing DMEM supplemented with 10 % FBS, 10 mmol/Lb-glycerol-phosphate (Merck, Darmstadt, Germany) 50 mg/mL ascorbic acid bi-phosphate (Sigma, St. Louis, MO, USA) and 100 nmol/L dexamethasone (Sigma, St. Louis, MO). After 21 days, HP-MSCs that undergone adipogenic differentiation were washed 3 times with PBS and fixed in 4 % paraformaldehyde for 30 min. Then, staining of HP-MSCs with Oil Red O stain (Sigma, St. Louis, MO, USA) was performed for detection of intracellular oil droplets and with Alizarin Red S stain (Sigma, St. Louis, MO, USA) for detection of calcium deposits.

Analysis of miR-146a-5p expression in HP-MSCs using RT-qPCR

Cells in the various groups were lysed using the TRIzol reagent and total RNA was purified using the miRNeasy mini kit (Qiagen, Hilden, Germany) based on the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using Hyper Script First Strand cDNA synthesis kit (Cat. No. 601-005, Seoul, South Korea) and RT primer specific for miR-146a-5p sequence. Following cDNA synthesis, preparation of PCR reaction was performed by adding high ROX SYBR Green master mix (Cat.No. 325402, Amplicon, Denmark) and 0.5 µl of each primer making a 10 µl volume mixture. RNU44 expression in cells was employed as the internal control and negative control reactions (NTC) was included that lacked template cDNA. All RT-qPCR reactions were performed in duplicate using step one plus Real-Time PCR System (Applied Biosystems, Foster City, CA). To assess the relative miR-146a-5p expression, the comparative $2^{-\Delta\Delta Ct}$ method was performed. The sequences of primers used for the analysis of miR-146a-5p expression in cells listed in Table 1.

Animal Studies

All procedures were approved by the ethics committee of the Tehran University of Medical Sciences (TUMS) (registration number: IR.TUMS.VCR.REC.1397.010). Also, all animal studies were performed in compliance with the guide for the care and use of laboratory animals. Male BALB/c mice weighing 19–22 g were purchased

from Royan Institute, Tehran, Iran. Experimental animals received human care and were maintained in a controlled environment (temperature 20±1 ˚C, humidity 40–70 % and 12:12 h light-dark cycle) in an animal facility with free access to water and food throughout the experiment unless as needed. The animals were divided into four experimental groups as follows: Cyclosporine A treated immunocompromised groups and immunocompetent groups without any immunosuppressive drug administration. Then, both groups were divided into direct intrahepatic and intraperitoneal xenotransplantation group. In the immunocompromised groups, the mice were treated with daily intraperitoneal administration of cyclosporine A (Novartis, New York, USA) diluted in normal saline at a dose of 10 mg/kg body weight from 24 h before cell transplantation until they sacrificed.

Direct intraparenchymal injection of HP-MSCs to liver tissue

BALB/c mice were anaesthetized using intraperitoneally infusion of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). The body temperature of the mice was maintained at 37 °C throughout the procedure. Abdominal surface of the mice body was shaved and cleaned with betadine and isopropyl alcohol for sterilization. For direct intrahepatic injection of cells, the anaesthetized mouse was maintained in a supine position. After median laparotomy under anaesthesia, 1×10^6 HP-MSCs suspended in 30 µl normal saline was injection into the median lobe of mice liver using a 31 gauge needle (BD, USA). The peritoneum was washed gently with normal saline for prevention of adhesion before closing the incision site with silk sutures. Recipient mice were left to recover and then were sacrificed at 24 h and day 28 after xenotransplantation.

Intraperitoneal injection of HP-MSCs

For injection of HP-MSCs to the intraperitoneal cavity of BALB/c mouse, 1×10^6 HP-MSCs were suspended in 200 µl PBS and injected intraperitoneally using 31 gauge needle (BD, USA).

Collection of xenotransplanted murine organs

Xenotransplanted BALB/c mice were sacrificed by cervical dislocation at predetermined time points, 24 h and 28 days after HP-MSCs injection. After animal death, various organs including liver, spleen, lungs and heart were collected for PCR analysis.

Polymerase chain reaction

Genomic DNA was extracted from the all or part of the various organs of the recipient mouse using the DNA extraction kit (Exgene Cell SV mini kit (106-101), geneall, Seoul, Korea) according to the manufacturer's instructions. Purity and concentration of genomic DNA extracts were assessed using a nanodrop. Genomic DNA extract from HP-MSCs alone or a mixture of lysed BALB/c hepatic tissue and HP-MSCs used as positive controls. Also, genomic DNA extract from hepatic tissue of BALB/c mice was analyzed for a murine specific sequence of β-actin as an internal control to avoid the false-negative results. Then, the presence of human-specific GAPDH sequence in DNA samples extracted from xenografted BALB/c mice organs and positive control samples was assessed with standard PCR assay. PCR reactions were prepared in a total volume of 25 µl with 12.5 µl of PCR master mix (Sinaclon, cat. No. MM206, Tehran, Iran) 1µl of each forward and reverse primers (10 pM) and 100 ng of template DNA. Sequences of primers pair specific for human GAPDH and mouse β-actin genes listed in Table 1. PCR conditions were accomplished by denaturation at 94 °C for 4 min followed by 45 cycles of amplification at 94 °C for 45 sec, 57 °C for 45 sec, and extension at 72 °C for 45 sec. The final extension step was extended at 72°C for 5 min. Non-template control (NTC) included in all tests to be sure the PCR procedure was free of any contamination. Then, PCR products were analyzed through 2 % agarose gel electrophoresis and DNA fragment observed using ultraviolet light.

Table 1. List of primers pair used in this study

Primer	**Sequence**
RNU44 forward primer	CCTGGATGATGATGATAGCAAATG
RNU44 universal reverse primer	TCGTATCCAGTGCAGGGTC
RNU44 stemloop	GTCGTATCCAGTGCAGGGTCCGACCGGTATTCGC ACTGGATACGACAGTCAG
mir-146a-5p-stemloop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC TGGATACGACAACCCA
mir-146a-5p	CGCCGATGAGAACTGTATTCCA
universal primer	CAAGTGAAGGGTCCGAGGTA
human GAPDH forward primer used for PCR	5' TCTTTGCAGTCGTATGG 3'
human GAPDH reverse primer used for PCR	5' TAGGGACCTCCTGTTTC 3'
Mouse β-actin forward primer	5' CTTCTTGGGTATGGAATCCTG 3'
Mouse β-actin reverse primer	5' GTGTTGGCATAGAGGTCTTTAC 3'

RESULTS

Differentiation and immunophenotyping of HP-MSCs

Results of HP-MSCs immunophenotyping using flow cytometry analysis demonstrated that expanded cells were positive for the MSC surface antigens CD44, CD73, CD90 and CD105 and were negative for CD34 and CD45. Also, after day 21 of adipogenic and osteogenic induction of HP-MSCs, detection of oil droplets within cells confirmed the

differentiation of HP-MSCs into adipocytes and observation of calcium deposits confirmed the differentiation of HP-MSCs into osteoblasts [22].

miR-146a-5p expression in HP-MSC

The RT-qPCR assay was used to identify miR-146a-5p expression in MSCs isolated from human placenta tissue. NT2 (NTERA-2, also designated NTERA2/D1, NTERA2, or NT2) cell line was selected as a positive control. Interestingly, RT-qPCR assay revealed that the levels of miR-146a-5p expression were higher than the negative control sample. Considering miR-146a-5p expression in HP-MSC, this study offers comprehensive data about HP-MSCs as a potential source of miR-146a-5p for miRNA-based therapy (Figure 1).

Animal mortality and morbidity

Most of BALB/c mice from intrahepatic group died due to surgical complications after 1 to 5 days post xenotransplantation. Thus, intrahepatic administration of cells using laparotomy was repeated for several times specially in cyclosporine a treated BALB/c mouse. No other complications or mortality were observed between intraperitoneally xenotransplanted BALB/c mice. Results of previous studies have confirmed that direct administration of therapeutic cells to hepatic parenchyma may facilitate more efficient engraftment of the cells into liver tissue than other routes. However, the results of the present study demonstrate that direct intrahepatic administration of cells to liver parenchyma is an invasive method and can lead to serious complications and animal death.

Assessment of HP-MSCs engraftment in mouse tissues

To detect human cells in mouse tissues using conventional PCR assay, positive controls were prepared by genomic DNA extract either from the mixture of HP-MSCs pellet and lysed hepatic tissues of BALB/c mice [23,24] or HP-MSCs pellet without mice tissue. Also, we assessed whether the GAPDH primer is truly specific for human placenta -derived MSCs or not. The results demonstrate the specific 174 bp band only in HP-MSCs or mixture of murine hepatic tissue and HP-MSCs as positive controls (Figure 2A). DNA extracts from a mouse tissue without xenotransplantation showed no specific band for human GAPDH sequence (Figure 2A). Also, DNA extract from hepatic tissue of the xenotransplanted BALB/c mice was assessed for the expression of a murine specific β-actin sequence as an

internal control to avoid false-negative results (Figure 2B).

Intraperitoneal administration of therapeutic cells is considered as a non-invasive approach to deliver stem cells into intraperitoneal organs such as liver as it is the least invasive method. Therefore, we adopted intraperitoneally cell infusion approach for xenotransplantation to BALB/c mice and infused a single dose of $1x10^6$ cells suspended in 150 µl PBS solution to the peritoneal cavity of either immunocompetent or cyclosporine A treated mouse. 24 h and 28 days after xenotransplantation, the animals were sacrificed and various organs including liver, spleen, lungs and heart were examined using PCR analysis for detection of human-specific GAPDH sequence. Unexpectedly, no human cells were found in liver, spleen, lungs and heart through PCR analysis. These findings suggest that intraperitoneally infused cells may not be translocated to the mouse organs in both immunocompetent and cyclosporine A treated mice. Indeed, in the immunocompromised mice, host immune suppression using cyclosporine a administration, may not result in the translocation and engraftment of HP-MSCs after 24 h and 28 days post xenotansplantation (Figure 3).

Also, direct intraparenchymal infusion of HP-MSCs to hepatic tissue of immunocompetent and cyclosporine A treated BALB/c mice fails to engraft in liver tissue. When HP-MSCs were directly xenotransplanted into the hepatic parenchyma of immunocompetent and cyclosporine a treated BALB/c mice through surgical intervention, no human cells were found 24 h and 28 days after cell delivery. Also, despite pretreatment of BALB/c mice using cyclosporine a injection, we found negative results of HP-MSCs xenotransplantation into the hepatic parenchyma of recipient mice (Figure 3). To screen for human cells in the xenotransplanted animals were analyzed the different samples for several times in each recipient. But, we could not detect the trace of human cells in murine organs. It is difficult to specify if HP-MSCs are cleared out from mouse tissues because of host immune responses or other causes such as low sensitivity of conventional PCR which lead to these unexpected results. The species difference between human and BALB/c mouse may lead to a decreased affinity of mouse T-cell receptors (TCRs) for human major histocompatibility complex (MHC) molecules [25]. Thus, one reason for the lack of evidence of human cells neither

through intrahepatic nor through intraperitoneal delivery may indicate the elimination of human cells by mouse antigen-presenting cells.

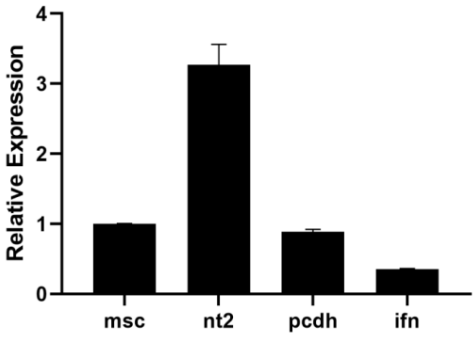

Figure 1. Expression pattern of miR-146a-5p in HP-MSCs. Total RNAs of HP-MSCs and control group were extracted and subjected into RT-qPCR reaction. RNU44 was used as an internal control. NT2 cell line was selected as a positive control. MSCs transduced with pCDH-IFN gamma-cGFP or empty pCDH-cGFP-puro vector were used as a negative control or control.

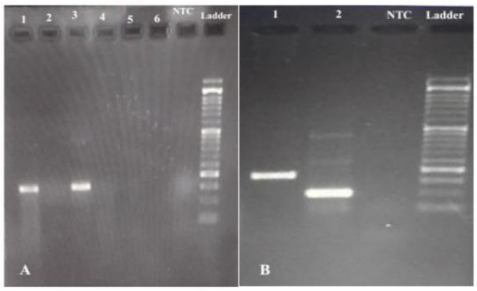

Figure 2. Images of agarose (2 %) gel electrophoresis for detection of human GAPDH specific band (amplicon size:174bp): (A) DNA isolated from HP-MSCs and mouse liver tissue injected with HP-MSCs were used as a positive controls and verification of primer pairs specific for human GAPDH sequence. DNA extracts from a mouse tissue without Xeno-transplantation showed no specific band for human GAPDH sequence (amplicon size: 174bp): Lane1: HP-MSCs, Lane 2: Mouse liver tissue without HP-MSCs transplantation. Lane 3: Mouse liver tissue injected with HP-MSCs, Lanes7: No template control (NTC), Lane 8: Marker 50bp. (B) DNA extract from HP-MSCs for detection of human GAPDH specific sequence (lane 1: 174bp) and DNA extract from mouse liver tissue for detection of murine specific β-actin sequence as an internal control (lane 2: 95bp). Lane 3: No template control (NTC), lane 4: Ladder 50bp.

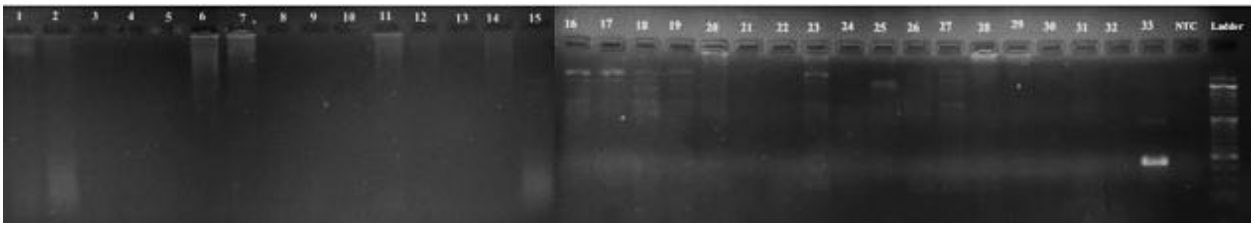

Figure 3. Agarose (2 %) gel electrophoresis was used to detect HP-MSCs in the xenotransplanted mouse organs in immunocompetent and cyclosporine A treated mouse. Human DNA was not detected in the liver, heart, lungs and spleen of different groups of xenografted BALB/c mouse. Lanes 1-32: mouse tissues injected with HP-MSCs, Lane 33: positive control, Lane 34: No template control (NTC), Lane 35: Ladder 50bp.

DISCUSSION

Although during the last years, great advances had been achieved in the field of cell-based miRNA therapy [9,26] several challenging issues including optimal cell delivery route need to be resolved. Deep understanding of the biodistribution and fate of infused therapeutic cells after various delivery techniques is essential to the progression and translation of this field of regenerative medicine to the clinic. Because of the ethical and rational restrictions about the dynamic behaviour of therapeutic cells in human body [27] there are many limitations related to the knowledge about the safety and efficacy of cell-based therapy products in human. Results of various studies have demonstrated that cell delivery through peripheral vein route is not ideal for transplantation of therapeutic cells despite easy and non-invasive entity of this modality because most of the administered cells entrapped in the lungs microvasculature due to the pulmonary first-pass effect [28].Additionally, it is suggested that cell delivery through portal vein allows targeted delivery of the therapeutic cells into the hepatic tissue [29]. But further studies have reported that this technique is a complicated procedure and could be resulted in portal vein thrombosis, hepatic failure and patient death [30]. To address these problems, some studies have suggested that administration of cells directly to liver parenchyma can be used as an efficient procedure for cell delivery and can lead to unlimited dissemination of infused cells throughout the liver tissue [31]. In spite of the enhanced transition of therapeutic cells in the liver parenchyma, one must examine the safety and engraftment of direct administration of cells to liver parenchyma for obtaining favorable therapeutic outcomes. Also, an alternative to these cell delivery techniques to liver tissue is through the intraperitoneal route. It might be a promising approach because of the minimally invasive nature of the technique and the potential for the high dose of cell delivery to the target organs within or outside of the peritoneal cavity [32]. Also, it has been hypothesized that administration of cells through the intraperitoneal cavity would be a more efficient strategy, as this technique hinders entrapment of cells in the lung microvasculature [33]. But until now, the biodistribution of injected cells through intraperitoneal delivery to liver parenchyma is not completely appreciated.

Xenotransplantation of various human cells to different animal models such as mouse are indispensable for further development of miRNA-based therapy to clinical use. Given the possible advantage of MSC as a miRNA delivery vehicle, in this study, we sought to examine the feasibility of MSCs application in cell based-miRNA therapy as a carrier to the hepatic tissue for designing of future studies using MSCs. For these purposes, in the present study, we used healthy BALB/c mice (n=6 per group) and investigate the biodistribution and fate of unmodified human MSCs after intraperitoneal and direct hepatic injection. To investigate the immunomodulatory effect of HP-MSCs in a xenograft model, these cells administered intraperitoneally and directly into the hepatic parenchyma of immunocompetent and cyclosporine A treated healthy mice. Then, cell translocation and retention were analysed using PCR technique in various tissues.

Although immunomodulatory properties of MSCs due to the expression of MHC class II and other co-stimulatory molecules was widely accepted, it became evident that xenotransplanted cells could be recognized and rejected by the host immune cells [34,35]. With respect to these issues and avoidance of consequent cell elimination by host immune cells, in our study xenotransplanted mouse model was treated daily using cyclosporine a regimen. Furthermore, we deliberately used xenotransplantation of HP-MSCs into the peritoneal cavity or hepatic parenchyma of immunocompetent mice model and compared them to cyclosporine a treated groups. In contrast to our expectations, findings of present work indicate that HP-MSCs that administered through intraperitoneal or direct intrahepatic routes cannot be successfully detected in the lungs, spleen, heart and liver of immunocompetent and cyclosporine A treated mice after 24 h and 4 weeks post-transplantation. Thus, xenograft rejection phenomena by host immune cells could be a possible explanation of these findings.

Considering host immune system responses against xenogeneic MSCs transplantation and lack of evidence of any human cells in the PCR analysis of four different groups of the mice in the present study, add to the growing body of evidence that various kinds of MSCs including HP-MSCs may not be immune-privileged as previous studies reported [36]. So, engraftment and biodistribution of xenogeneic human MSCs in our study maybe hampered, associated with activation and proliferation of host immune cells against exogenous donor cells and this result could interpret the lack

of traceability of transplanted HP-MSCs in our study. Also, the results of the present study suggest that biodistribution and engraftment of xenogeneic HP-MSCs in the immunocompetent mice as a xenograft require effective immunosuppressive regimen other than cyclosporine to hamper immune cell activation and consequently xenograft rejection. Our results are partly in accordance with the study of Bazhanov *et al.* who reported that human bone marrow-derived MSCs could form aggregates with mouse immune cells including macrophages and $B220^+$ lymphocytes in the peritoneal cavity. Thus, aggregated cells adhered to the peritoneal membranes and could not be translocated to the other organs [32]. A novel aspect of our study in comparison with Bazhanov *et al*. study is the possible immunogenicity of xenografted placenta-derived MSCs that previous works reported immune-privileged characteristics for them [36].

Negative results of cell engraftment in mouse hepatic tissue may be in contradictory with previous reports that cell-based therapies improved hepatic structural and functional recovery in xenotransplanted models using human bone marrow-derived MSCs [37]. Parameters including the use of immunodeficient or immunocompromised model have been reported to highly influence engraftment and fate of transplanted cells and consequently improvement of target organs. Under our experimental conditions, no differences in cell engraftment and retention were detected between immunocompromised and cyclosporine A treated groups. In addition to the host immune rejection of xenotransplanted cells, another reason for the negative results of cell engraftment in hepatic tissue may be due to the low sensitivity of the PCR method. Techniques such as MRI are widely used in cell monitoring experiment but suffer from problems such as low sensitivity, the requirement to physical cell labeling and false-positive results due to the engulfment of administered cells by macrophages [38,39]. Also, nuclear imaging technique including positron emission tomography (PET) and Single Photon Emission Computed Tomography (SPECT) have a low resolution at a cellular level and radioisotopes that routinely used for cell labeling possess a limited half-life [38].

Based on previous reports, the PCR technique offers a specific and sensitive detection of administered human cells in xenograft models [40]. This technique is approximately simple, normalisable [40] and without the requirement of cell labeling

or genetic manipulation of administered cells [41]. Furthermore, according to the previous studies, despite the lack of knowledge about the spatial scheme of cell biodistribution in the target tissue, PCR technique is considered as the most favourable, cost-effective and sensitive method for tracking of infused cells *in vivo* [42]. Although based on the previous reports, the PCR method works well for *in vivo* cell engraftment and fate assay [40], its sensitivity and accuracy should be addressed in future xenotransplantation studies. Also, whether the observed negative results of cell engraftment are also associated with the small size of tissue samples used for DNA extraction from fresh tissue remains an open question for future investigations. Previous reports have demonstrated that beneficial effect of MSCs transplantation on organ function and regeneration can be detected while the percent of infused cells present in the damaged tissue is very low or negligible. These findings indicate that other phenomena than cellular Integration and engraftment contribute to organ healing and regeneration [43]. Moreover, accumulating evidence indicate that the therapeutic potential of MSCs in orchestrating tissue regeneration after cell infusion, mediated through MSCs paracrine signaling [44]. Based on previous reports the secretion of Extracellular Vesicle (EV) from various type of MSCs mediate paracrine signaling of these cells by carrying bioactive factors such as miRNA to the target tissue [45].

In this study, we selected miR-146a-5p because among the various microRNAs, expression of miR-146a-5p was considerably decreased in activated hepatic stellate cells obtained from nonalcoholic fibrosing steatohepatitis samples. Overexpression of miR-146a-5p in activated hepatic stellate cells inhibit collagen deposition and suppress Wnt signaling pathway. Consequently, these processes lead to improvement of liver fibrosis. Indeed, miR-146a-5p delivery might contribute as a suitable regulator in the pathogenesis liver fibrosis [10]. In our study miR-146a-5p was introduced through viral transduction (unpublished data). However, after transduction we did not observe significant differences in the expression of miR-146a-5p between transduced mesenchymal stem cells and naive mesenchymal stem cells. Therefore, we decided to track naive mesenchymal stem cells that injected through various delivery routes into healthy mice and results of this manuscript are based on the cell tracking studies using naive

mesenchymal stem cells. In conclusions, considering the miR-146a-5p expression in naive HP-MSCs together with negative results of cell detection in hepatic tissue, the present study suggests the cell-free secretome based miR-146a-5p delivery for modulation of altered miRNA expression in activated hepatic stellate cells. Also, secretome based miR-146a-5p delivery to activated hepatic stellate cells may eliminate the risk of xenograft HP-MSCs rejection by host immune cells [46]. Indeed, MSCs' secretome-mediated miRNA therapy may has therapeutic potential in treating liver fibrosis through MSCs paracrine effect irrespective of direct translocation and retention of infused cells in hepatic tissue.

CONCLUSION

Considering the miR-146a-5p expression in HP-MSCs together with negative results of cell engraftment in hepatic tissue, the present study suggests the cell-free secretome-based miR-146a-5p delivery for modulation of altered miRNA expression in hepatic stellate cells for the treatment of liver fibrosis.

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