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microRNA-27b suppresses mouse MSC migration to the liver by targeting SDF-1α *in vitro*

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ABSTRACT

The SDF-1/CXCR4 axis is critical for inducing stem cell mobilization into the circulation, for homing stem cells to the site of injury, and for stem cell participation in the regeneration of liver tissue. In this study, we have gained insight into the molecular mechanisms involved in regulating the expression of SDF-1α by miRNAs. Using microarray and bioinformatics approaches, we identified six miRNAs with differential expression in damaged liver tissue (21 days after liver injury) compared to normal C57BL/6 murine liver tissue and further confirmed these observations by qPCR; miR-23a, which was identified by other researchers, was also included for comparative purposes. We found that miR-23a, miR-27a and miR-27b expression was significantly lower in the damaged liver than in the normal liver ($p < 0.05$). We further confirmed that miR-27b could directly interact with the 3′UTR of SDF-1α to suppress SDF-1α protein expression using a luciferase reporter assay and Western blot analysis. In addition, we found that the over-expression of miR-27b significantly reduced the directional migration of primary cultured CRCX4-positive murine mesenchymal stem cells (mMSCs) *in vitro* using a transwell assay. These results suggest that miR-27b may be a unique signature of the stem cell niche in the damaged mouse liver and that mir-27b can suppress the directional migration of mMSCs by down-regulating SDF-1α expression by binding directly to the SDF-1α 3′UTR.

1. Introduction

End-stage liver disease (ESLD) is the final stage of acute and chronic liver disease and is often accompanied by liver failure [1]. The traditional treatment of ESLD is relatively ineffective and is associated with high mortality. Recently, the transplantation of mesenchymal stem cells (MSCs) has been considered to be a potentially effective treatment for ESLD due to the ease of isolation, the rapidity of expansion, and the differentiation capacity of MSCs [2]. It is not yet clear how MSCs are recruited to injured liver tissue and recover its function? It has been demonstrated that stromal cell-derived factor-1α (SDF-1α), also called CXCL12, plays a critical role in the migration, chemotaxis, homing and transdifferentiation of MSCs [3]. In myocardial infarction, the increase of SDF-1α at the site of injury is able to induce the chemokine receptor CXCR4-positive MSCs to the infarction area [4]. In our previous studies, the SDF-1α level had been found to reach a peak on the 21st day after liver injury, and mMSCs chemokine expression by the damaged liver and the transdifferentiation of hepatocyte-like cells also peaked at the same time [5]. An increase in SDF-1α expression during the early stages of liver injury could help MSCs home to damaged liver tissue and contribute to tissue repair earlier.

Although SDF-1α plays an important role in the repair of damaged tissue through the post-traumatic mobilization of MSCs, the mechanism regulating the release of SDF-1α remains unclear. Therefore, enhancing endogenous SDF-1α release in damaged liver tissue through genetic regulation could be a clinically effective means of inducing MSC adhesion and migration into the injured liver parenchyma.

Until now, most research has concentrated on the regulation of SDF-1α at the transcriptional level, and studies have shown that c/EBPβ binding to a specific response element located at −1171 in the SDF-1α promoter region drives the transcription of the SDF-1α gene in response to different stimuli [6]. The non-coding RNA-mediated post-transcriptional regulation of SDF-1α, however, remains to be fully elucidated.

Abbreviations: ESLD, end-stage liver disease; mMSCs, murine mesenchymal stem cells; SDF-1α, stromal cell-derived factor-1α; 3′UTR, 3′-untranslated region.

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MicroRNAs (miRNAs) are a class of small non-coding RNAs that function to control gene expression by inducing the degradation or inhibiting the translation of an mRNA through an association with its 3′-untranslated region (3′UTR) [7,8]. Accumulating evidence has shown that endogenous miRNAs are broadly exploited to regulate a variety of proteins. Thus, miRNAs might regulate the expression of SDF-1α and further affect MSC migration into damaged tissue in the early stages of liver injury by impairing SDF-1α expression. This might provide a theoretical basis for stem cell transplantation in the treatment of ESLD.

In this study, 97 predicted murine miRNA binding sites within the SDF-1α 3′UTR were identified in silico using microma (www.microma.org). Then, a microarray was performed to detect 37 miRNAs that were differentially expressed in burned murine skin compared to the surrounding normal skin. Five of these miRNAs were among the SDF-1α-regulating miRNAs predicted by microarray analysis (Supplementary Fig. 1) and bioinformatics. For comparative analysis, miR-23a, which was identified by other researchers [9] but was not among the five miRNAs identified by our methods, was also included.

We investigated the effects of the six miRNAs that were differentially expressed in burned murine skin and the surrounding normal skin to gain insight into the biological functions of SDF-1α-regulating miRNAs in mouse liver injury, and we identified SDF-1α as a target of post-transcriptional regulation by these miRNAs.

2. Materials and methods

2.1. MSC culture

MSCs isolated from male C57BL/6 mice were generously gifted by Dr. Guangping Liang (Institute of Burn Research, Third Military Medical University, China). They were identified as CD44+, CD29+ and SCA-1+ but CD117− [10] by flow cytometry analyzes, and they were capable of differentiating into adipocytes, chondrocytes, and osteoblasts *in vitro* [11] (Supplementary Fig. 2). The MSCs were cultured routinely in α-MEM (GiBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GiBCO, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Construction of the acute liver damage model

A total of 40 male C57BL/6 mice were obtained from the DaPing Hospital Animal Center (Third Military Medical University, China). They were injected with CCl_4 [12] (Sigma, USA) in the abdominal cavity at a dose of 15 μl/g body weight of 0.3% (v/v) CCl_4 dissolved in peanut oil (Shandong Luhua Group, China). All mice were approximately 6 weeks old, weighed between 18 and 22 g, and were bred under pathogen-free conditions.

2.3. Expression of SDF-1α in the damaged mouse liver tissue detected by ELISA

SDF-1α was measured in the liver tissue of C57BL/6 mice on the 1st, 2nd, 3rd, 4th, 7th, 21st, and 28th days after acute liver injury using a standard enzyme-linked immunosorbent assay (ELISA) (RayBiotech, Norcross, USA) according to the manufacturer's protocol [13]. The concentration of SDF-1α was determined using a DENLEY DRAGON Wellscan MK3 instrument (Thermo, USA).

2.4. Detection of miRNAs by real-time qPCR

Total RNA was isolated from damaged liver tissue of C57BL/6 mice (21st day after injury) and from normal liver tissue using RNAiso plus (TAKARA, Japan) and was quantified using an ultraviolet spectrophotometer (BECKMAN DU-600, USA). First-strand cDNA was synthesized from 500 ng total RNA by reverse transcription using a gene-specific primer (GSP). Real-time PCR using SYBR Premix EX Taq™ II (TAKARA, Japan) was performed according to the following parameters: 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s; and a final extension step at 78 °C for 32 s. A melting curve from 60 to 95 °C (in incre-

Table 1
Primer pairs used for real time q-PCR.

Gene name	GenBank™	Primers
U6	NM_001204274.1	F:GCTTCGGCAGCACATATACTAAAAT R:CGCTTCACGAATTTGCGTGTCAT
mmu-miR-1	NR_029528.1	GSP:GGGGTGGAATGTAAAGAAGT R:CAGTGCGTGTCGTGGAGT
mmu-miR-136	AJ 459747.1	GSP:GGAACTCCATTTGTTTTGA R:CAGTGCGTGTCGTGGAGT
mmu-miR-214	NR_029796.1	GSP:GACAGCAGGCACAGACA R:TGCGTGTCGTGGAGTC
mmu-miR-23a	NR_029740.1	GSP:CCATCACATGCCAGG R:CAGTGCGTGTCGTGGAGT
mmu-miR-27a	NR_029746.1	GSP:GGGGTTCACAGTGGCTAA R:CAGTGCGTGTCGTGGAGT
mmu-miR-27b	NR_029531.1	GSP:GGGGTTCACAGTGGCTAAG R:CAGTGCGTGTCGTGGAGT

GSP, gene specific primer; F, forward primer; R, reverse primer.

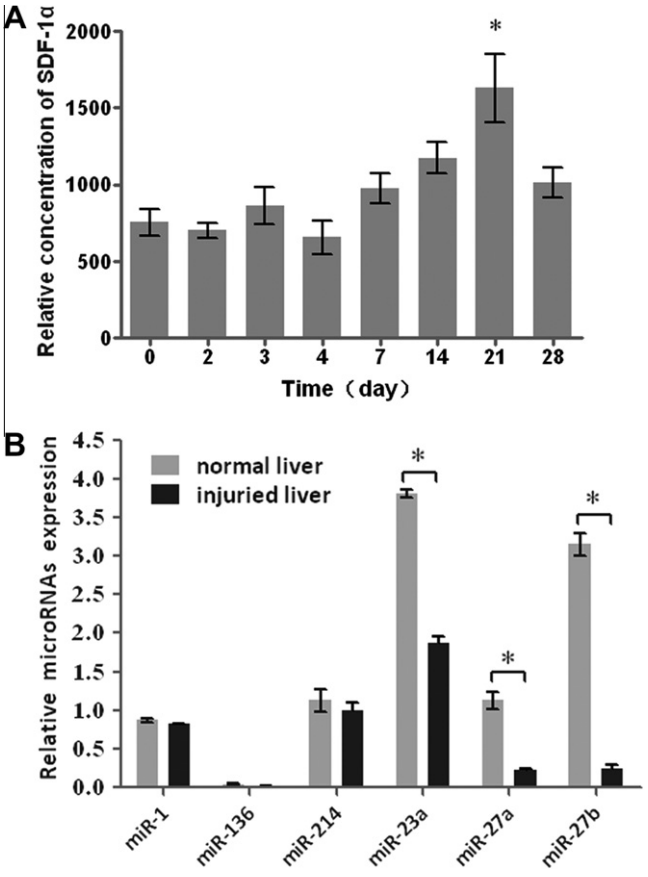

Fig. 1. The expression of SDF-1α and different miRNAs in the damaged liver. (A) The expression of SDF-1α in damaged liver tissue at different time points, as measured by ELISA. The concentration of SDF-1α increased slowly in damaged liver tissues and reached a peak on the 21st day after acute liver injury ($n = 3$, $^*p < 0.05$; Mann–Whitney test). The error bars represent SD. (B) The expression of different miRNAs in the damaged liver tissue on the 21st after liver injury, as measured by qPCR ($n = 3$, $^*p < 0.05$; Wilcoxon marched pair test). The error bars represent SD.

ments of 0.5 °C/5 s) was also performed. The fluorescence threshold value (Ct) was calculated using the 7500 qPCR System (ABI, USA). U6 was used as an internal standard of miRNA expression for normalization [14]. The relative quantity (RQ) of different miRNAs was calculated as $RQ = 2^{-\Delta\Delta Ct}$. The gene-specific primer pairs used are listed in Table 1.

2.5. Lentiviral construct design and sorting of infected cell

Lentiviral vectors containing pre-miRNAs and a GFP reporter under the control of the CMV promoter were purchased from GeneChem Management Inc., Shanghai, China. The lentiviral vectors containing the miRNAs were named LV-*mmu-mir*-1 (LV-*mir*-1), LV-*mmu-mir*-136 (LV-*mir*-136), LV-*mmu-mir*-214 (LV-*mir*-214), LV- *mmu-mir-23a* (LV-*mir-23a*), LV-*mmu-mir-27a* (LV-*mir-27a*), LV-*mmu-mir*-27*b* (LV-*mir*-27*b*), and LV-*cel-mir*-67 (negative control). mMSCs were infected with these lentiviruses at a multiplicity of infection (MOI) of 10, and mMSCs infected with GFP proteins were sorted by flow cytometry (FCM) (FACSAria Cell sorter, BD, USA) using laser excitation at 488 nm after transfection for 96 h. (Fig. 4A and B). The infection efficiency was nearly 100%, as assessed by laser scanning confocal microscopy (Leica, Germany) (Fig. 2A).

2.6. Western blot

Total protein was extracted using RIPA lysis buffer (Sangon Biotech, China). The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China). The protein extracts were separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore, USA). Non-specific protein was blocked with 5% dried skim milk for 1 h, and the membranes were incubated overnight with antibodies directed against SDF-1 α (1:500, eBioscience, USA) and GAPDH (1:3000, Santa Cruz, USA). Horseradish peroxidase-conjugated secondary antibodies (Zhong-Shan, China) were diluted at 1:5000. The signals were assessed using ECL reagents (Thermo, USA) and a Gel-DOC 2000 imaging scanner (BIO-RAD, USA) [14].

2.7. Luciferase reporter assay

The full-length SDF-1α 3′UTR was amplified from mouse cDNA. The amplified wild-type fragment cloned into the DraI and XbaI sites of the pmirGLO dual-luciferase miRNA target expression vector (Promega, USA) was named W for short. The three nucleotides complementary to the SDF-1α-regulating miR-27b seed region in the PmirGLO-SDF-1α 3′UTR were mutated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The mutated reporting vector was named M for short. All of vectors were verified by DNA sequencing. HEK293 cells (a cell line optimized to assess miRNA function) were co-transfected with 100 ng of the luciferase reporter vector and 100 nM of the miRNA mimic or the negative control (RIBOBIO, China) in 96-well plates using Lipofectamine 2000 (Invitrogen, USA) [14]. After 24 h of transfection, Firefly and Renilla luciferase activities were measured using the dual-luciferase assay (Promega, USA).

2.8. In vitro chemotaxis assay

The SDF-1α-regulating effects of each miRNA on MSC migration was determined using a Transwell Chamber assay (Corning, USA). MSCs were trypsinized and resuspended in α-MEM containing 2.5% (v/v) FBS at 1×10^5 cells/ml, and 100 μl of this cell suspension was added to the upper chamber. MSCs infected with either SDF-1α-regulating miRNA or cel-mir-67 (as a negative control) were trypsinized and resuspended in α-MEM containing 2.5% (v/v) FBS

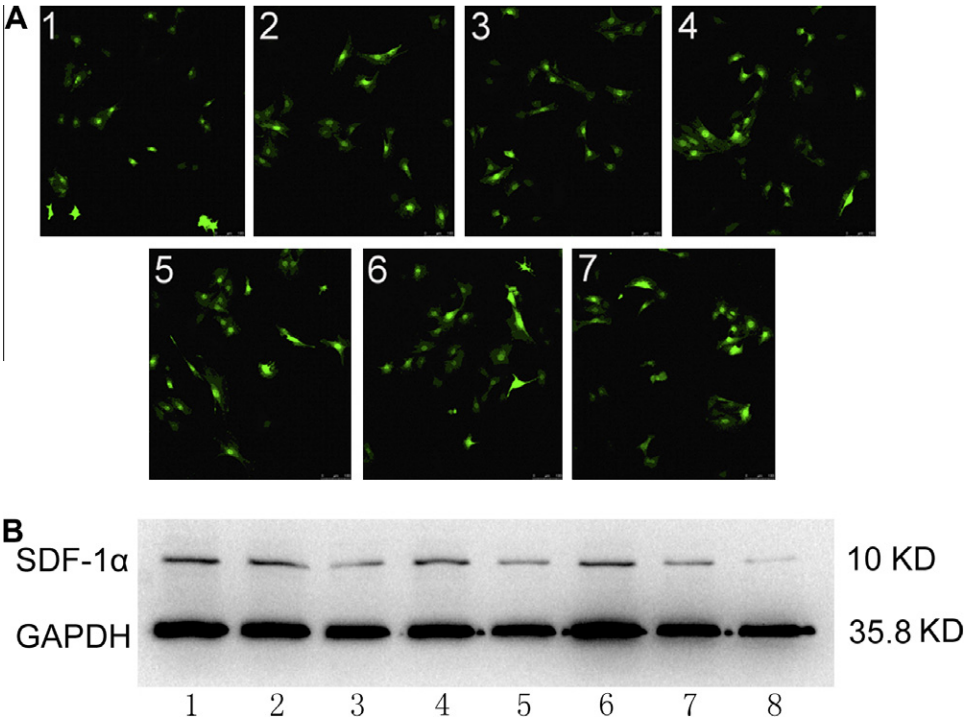

Fig. 2. The expression of SDF-1α in MSCs infected with different miRNAs. (A) MSCs infected with different lentiviral vectors expressing miRNAs and GFP detected by laser scanning confocal microscopy. 1, LV-*mir*-1; 2, LV-*mir*-136; 3, LV-*mir*-214; 4, LV-*mir-23a*; 5, LV-*mir*-27*a*, 6, LV-mir-27*b*; 7, LV- *mir*-67 (negative control). (B) The expression of SDF-1α protein in MSCs after infection with lentiviral vectors expressing miRNA for 96 h detected by Western blot. MSCs infected with miR-27b exhibited significantly lower SDF-1α expression than did MSCs infected with LV-cel-miR-67 or uninfected MSCs. Lane 1, MSCs; lane 2, MSCs/LV-cel-miR-67; lane 3, MSCs/LV-miR-1; lane 4, MSCs/LV-miR-136; lane 5, MSCs/LVl-miR-214; lane6, MSCs/LV-miR-23a; lane 7, MSCs/LV- miR-27a; lane 8, MSCs /LV-miR-27b.

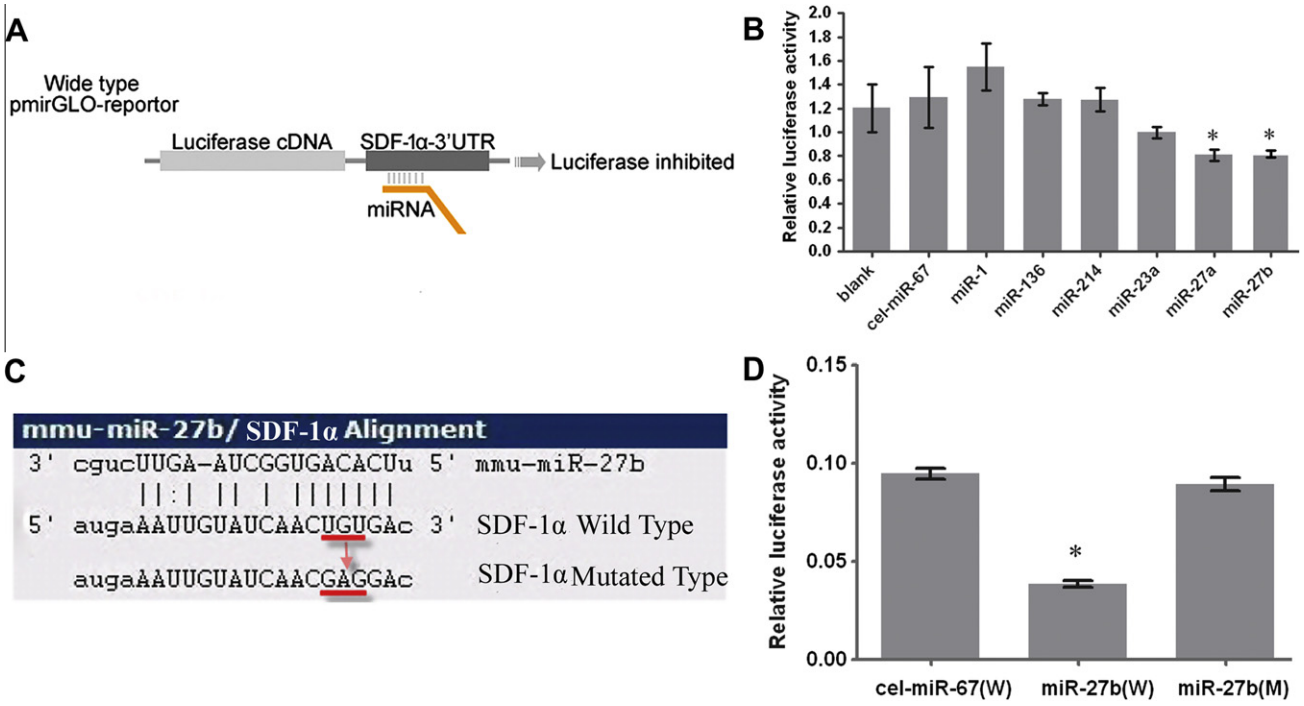

Fig. 3. miR-27b targets the SDF-1α 3′UTR, as shown in a luciferase reporter assay. (A) Potential binding sites for miRNAs in the 3′UTR of SDF-1α predicted by bioinformatics. (B) The activities of the Firefly and Renilla luciferases were assayed in HEK293 cells 24 h after co-transfection with different miRNAs and the SDF-1α 3′UTR ($n = 3$, $^*p < 0.05$; Mann–Whitney test). The error bars represent SD. (C) The predicted miR-27b binding sites in SDF-1α were mutated by site directed mutagenesis in the miRNA-27b seed region. (D) The activities of the Firefly and Renilla luciferases were assayed in HEK293 cells 24 h post co-transfection with different miRNAs and the mutated SDF-1α 3′UTR ($n = 3$, $^*p < 0.05$; Mann–Whitney test). The error bars represent SD.

at 1×10^5 cells/ml, and 600 μl of this cell suspension was added to the lower well. After 24 h of incubation at 37 °C in a humidified atmosphere containing 5% CO_2, the cells on the top of the membrane were removed, and the cells on the bottom of the membrane were fixed for 30 min in 4% paraformaldehyde, followed by staining with 0.5% (w/v) crystal violet (Beyotime, China) for 45 min. The cells in five random fields were counted under a light microscope at 40× magnification (Olympus, Japan). These experiments were performed in triplicate [14].

2.9. Statistical analysis

All data are presented as the means ± SD. The significance of the differences between groups was determined with the Wilcoxon matched pair test or Mann–Whitney test. A value of $p < 0.05$ was considered statistically significant. $^*p < 0.05$. All statistical analyzes were carried out with Prism 5.0 software (GraphPad).

3. Results

3.1. Expression of SDF-1α in the damaged murine liver tissue

Acute liver injury was induced in C57BL/6 mice by CCl_4 injection into the abdominal cavity. The mice were sacrificed for liver removal on the 1st, 2nd, 3rd, 4th, 7th, 21st, 28th days of acute liver injury to allow SDF-1α detection by ELISA. The concentration of SDF-1α increased slowly in damaged liver tissues and reached a peak on the 21st day after acute liver injury ($p < 0.05$) (Fig. 1A).

3.2. miRNAs that are down-regulated in damaged liver tissue were identified by qPCR

The down-regulation of SDF-1α-regulating miRNAs in damaged liver tissue (from C57BL/6 mice after the 21st day of liver injury) compared to normal murine liver tissue was confirmed by qPCR. The expression of miR-23a, miR-27a and miR-27b were significantly lower in the damaged liver than in the normal liver ($p < 0.05$) (Fig. 1B). This result suggests that these 3 miRNAs may be involved in the regulation of SDF-1α at the post-transcriptional level.

3.3. miR-27b decreased SDF-1α protein expression

Proteins are responsible for the execution of many functions in biological development. To determine whether the overexpression of the above six miRNAs suppressed the level of endogenous SDF-1, we performed Western blots to investigate the SDF-1 protein levels in mMSCs that had been infected with miRNA lentiviral vectors for 96 h. As shown in Fig. 2B, the miRNAs miR-1, miR-214, miR-27a, and miR-27b reduced the level of SDF-1α protein. In particular, miR-27b suppressed the expression of SDF-1α protein in MSCs significantly compared to cel-miR-67 (negative control). This result confirmed that miR-27b could negatively regulate the expression of SDF-1α at the protein level, although the underlying mechanism remains unclear.

3.4. miR-27b regulated SDF-1α by interacting with the 3′UTR of SDF-1α

The tested miRNAs were predicted to target the SDF-1α 3′UTR via separate binding sites using a bioinformatics approach. To confirm that these 6 miRNAs bound to the predicted sites, the entire SDF-1α 3′UTR was cloned into the pmirGLO dual-luciferase reporter vector (Fig. 3A). Subsequent luciferase reporter assays showed dramatically lower expression of Firefly/Renilla luciferase in the presence of miR-27a and miR-27b than in the presence of a negative control and a blank control (Fig. 3B). Because we had found that miR-27b greatly suppressed the posttranslational (i.e., pro-

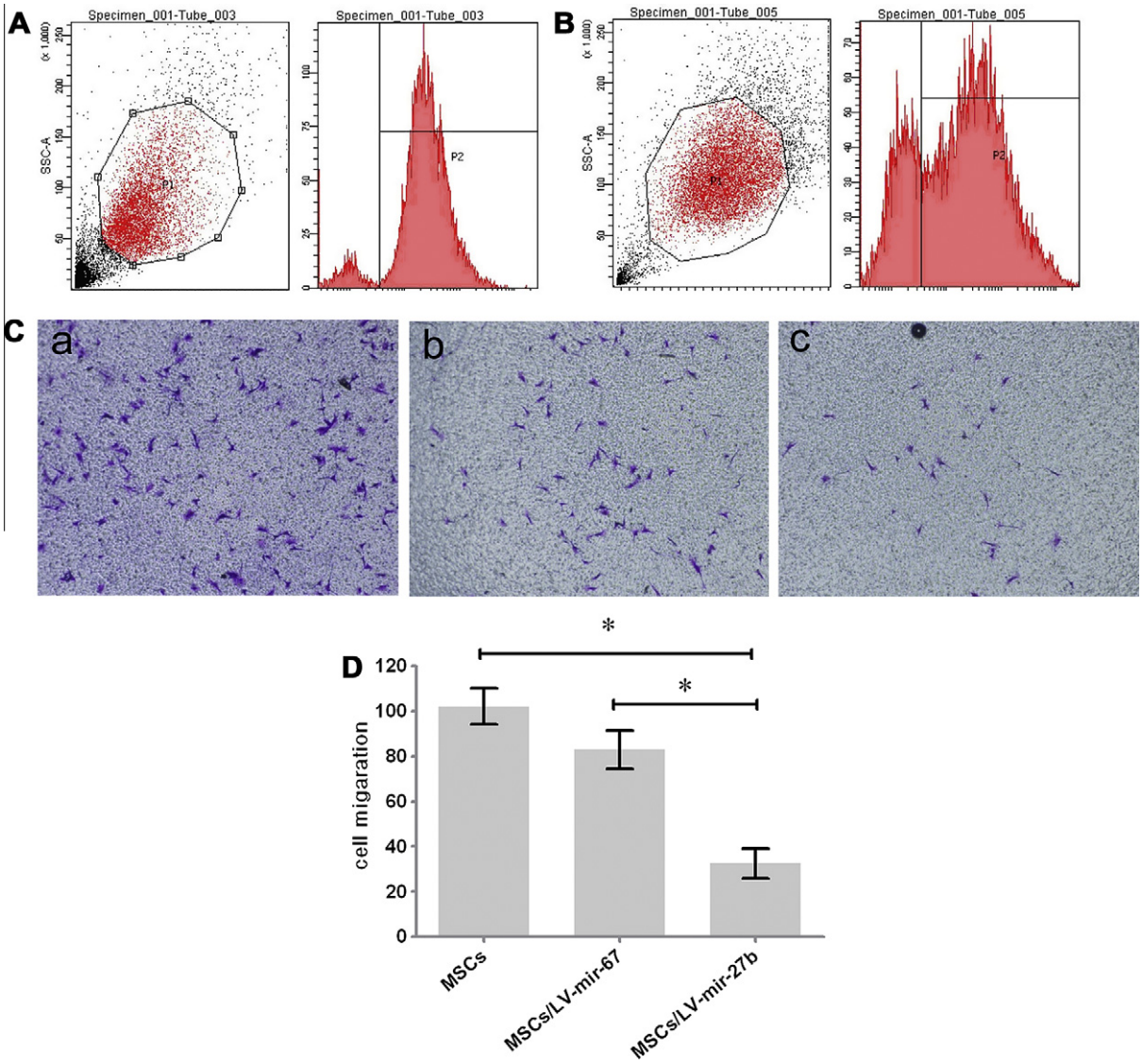

Fig. 4. The effect of miR-27b on MSC migration. (A) Flow cytometry-sorted MSCs infected with LV-cel-miR-67 after transfection for 96 h (40× magnification; light microscopy) to ensure the infection efficiency of sorted MSCs was high. (B) Flow cytometry-sorted MSCs infected with LV-miR-27b after transfection for 96 h to ensure the infection efficiency of sorted MSCs was high. (C) The migration capacity of MSCs over-expressing miR-27b was assessed with a transwell migration assay. a, MSCs; b, MSCs/LV-cel-miR-67; c, MSCs/LV-miR-27b. (D) Alteration of the chemotactic capacity of MSCs in different niches ($n = 3$, $^{*}p < 0.05$; Mann–Whitney test). The error bars represent SD.

tein) level of SDF-1α, we mutated the miR-27b binding site in the SDF-1α-3′UTR using site-directed mutagenesis (Fig. 3C). The results of the luciferase reporter assays using this construct showed that the mutation of seed sequences in PmiGLO-SDF-1α-M-3′UTR completely eliminated the inhibitory effect of miR-27b (Fig. 3D). This result further confirmed that miR-27b is able to inhibit SDF-1α translation by interacting directly with the SDF-1α 3′UTR.

3.5. miR-27b inhibits mMSC directional migration in vitro

We next examined whether miR-27b could influence the migration of mMSCs in culture. The overexpression of miR-27b in mMSCs reduced SDF-1α secretion and significantly inhibited the recruitment and homing of other normal MSCs compared to the negative controls *in vitro* ($p < 0.05$) (Fig. 4C and D).

4. Discussion

Recent studies have demonstrated that the SDF-1/CXCR4 axis is critical for inducing stem cell mobilization into the circulation, for homing stem cells to the site of injury, and for stem cell participation in the regeneration of liver tissue [15]. However, stem and progenitor cells migrate to their niches in injured sites along a gradient of SDF-1α, the levels of which increase slowly after injury. We found that the level of SDF-1α reached a peak on the 21st day after liver injury, and MSC homing to the damaged liver and transdifferentiation into hepatocyte-like cells peaked at the same time. Thus, increasing SDF-1α release during the early stages of liver injury may be helpful to promote MSC homing to damaged liver tissue and contribute to more rapid liver repair.

To gain insight into the molecular mechanisms involved in regulating the expression of SDF-1α, we investigated the possibility that SDF-1α may be subject to post-transcriptional regulation by miRNA [14]. Different stem cells and somatic tissues have unique miRNA signatures [16,17]. These tissue-specific miRNAs affect stem cell self-renewal, differentiation, and chemotaxis, and they also change the cellular components of their niche. We hypothesized that liver tissues with different levels of SDF-1α expression could be compared to identify differentially expressed miRNAs that might be responsible for controlling SDF-1α expression [18].

Based on microarray and bioinformatics analyzes, we identified 6 miRNAs that were differentially expressed in damaged liver tissue (21 days after liver injury) and normal liver tissue in C57BL/6 mice and further confirmed their differential expression by qPCR; miR-23a, which was identified by other researchers, was also included for comparative purposes. We found that miR-23a, miR-27a and miR-27b levels were significantly lower in the damaged liver than in normal liver tissue ($p < 0.05$). The levels of SDF-1α expression also increased in the damaged liver after acute injury. We therefore used a luciferase reporter assay to determine whether any of these 6 miRNAs interacted directly with the 3′UTR of SDF-1α to suppress SDF-1α translation. Our data suggested that miR-27b and miR-27a mediated a significant reduction of SDF-1α ($p < 0.05$); however, the other miRNAs had minor or no effects on SDF-1α protein levels.

Having established that these miRNAs can repress SDF-1α, we next demonstrated that the over-expression of these miRNAs reduces endogenous SDF-1α expression in MSCs. It was recently reported that SDF-1α is expressed in MSCs, as is its cognate receptor, CXCR4 [19]. Thus, the interaction between SDF-1α and CXCR4 in MSCs may activate an intracellular mechanism that promotes MSC mobilization from the stem cell pool into circulation [20]. SDF-1α translation was repressed most significantly by miR-27b (Fig. 2B), but the over-expression of miR-1, miR-214, and miR-27a also suppressed SDF-1α protein expression to different degrees (Fig. 2B). However, SDF-1α protein expression was not repressed in the context of miR-1 or miR-214 overexpression, despite the results of the luciferase reporter assay (Fig. 3B). These data illustrate the limitations of bioinformatic prediction algorithms for discerning potentially biologically significant miRNA binding sites within mRNAs [19]. We performed further mutation studies of the putative miRNA binding sites predicted by bioinformatics and determined that the SDF-1α mRNA is a direct target of miR-27b (Fig. 3D). We found that SDF-1α was highly expressed in the murine liver, where it was secreted by diverse cell types (Supplementary Fig. 3); in fact, mMSCs were thought to be one of the major sources of SDF-1α [21]. Meanwhile, the expression of miR-27b was significantly reduced in the injured murine liver. These results raise the possibility that miR-27b plays an important role in regulating SDF-1α to inhibit the homing and engraftment of MSCs. We further used a transwell migration assay to demonstrate that miR-27b was able to reduce SDF-1α secretion to inhibit the recruitment and homing of other normal MSCs *in vitro*.

In addition, we hypothesize that the inhibition of miR-27b will be shown to allow the expression of SDF-1α to stimulate the homing and directional migration of MSCs, and that miRNAs could serve as a biological rheostat, depending on the context [22] and source of the external stimulus. Furthermore, recent studies have demonstrated that miR-27 activates an angiogenic switch by promoting the endothelial tip cell fate and by sprouting to promote venous differentiation [23] and targets PPARγ to inhibit tumor progression and the inflammatory response in neuroblastoma cells [24]; furthermore, miR-27b expression increases during human breast cancer progression [25]. These data highlight the complexity of the biological effects mediated by mir-27b and indicate the existence of additional unknown mir-27b target genes that remain to be explored.

In conclusion, we have demonstrated that mir-27b suppresses the directional migration of MSCs by down-regulating SDF-1α expression. These observations further contribute to our understanding of how SDF-1/CXCR4 interactions modulate cell behavior and may provide a novel therapeutic approach to promote MSC homing to damaged liver tissue and contribute to more efficient tissue repair. In addition, we are currently assessing the possibility that miR-27b may be a unique signature of damaged liver tissue in the mouse.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.027.

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