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Upregulation of PIM2 by Underexpression of MicroRNA-135-5p Improves Survival Rates of Skin Allografts by Suppressing Apoptosis of Fibroblast Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: It has been reported that miR-135-5p is involved with many diseases. In this study, we aimed at define the relationship between miR-135-5p level and burn patient survival after skin transplantation.

Material/Methods: Expression of miR-135-5p and PIM2 was measured using real-time PCR and Western blot analysis in the skin samples collected from burn patients who received skin graft or in the fibroblast cells transfected with miR-135-5p mimics or inhibitors. The regulatory association between miR-135-5p and PIM2 was verified using bioinformatics analysis and luciferase assay.

Results: The expression level of miR-135-5p was determined in 60 tissue samples divided into 2 groups based on the presence of rejection (long survival n=30, and short survival n=30). We found that miR-135-5p was substantially downregulated in the long survival group. We then searched the miRNA database online with the "seed sequence" located within the 3'-UTR of the target gene, and then validated PIM2 to be the direct gene via luciferase reporter assay system. We also established the negative regulatory relationship between miR-135-5p and PIM2 via studying the relative luciferase activity. We also conducted real-time PCR and Western blot analysis to study the mRNA and protein expression level of PIM2 among different groups (long survival n=30, short survival n=30) or cells treated with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors, indicating the negative regulatory relationship between MiR-135-5p and PIM2. We also conducted experiments to investigate the influence of miR-135-5p and PIM2 on viability and apoptosis of cells. The results showed miR-135-5p reduced the viability of cells, while PIM2 negatively interfered with the viability of cells, and miR-135-5p inhibited apoptosis and PIM2 suppressed apoptosis.

Conclusions: MiR-135-5p is involved with the prognosis of burn patients after skin transplantation. PIM2 is a virtual target of miR-135-5p, and there is a negative regulatory relationship between miR-135-5p and PIM2. MiR-135-5p and PIM2 interfered with the viability and apoptosis in cells.

MeSH Keywords: **Allografts • Apoptosis • Cell Survival • MicroRNAs**

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Background

Patients with large cutaneous wounds and burns usually do not have adequate donor site tissue for skin autograph to cover all affected lesions. Available techniques include cultured epidermal autografts, synthetic materials, and cadaveric allograft for large cutaneous wounds and burns. There is often complicated infection associated with synthetic materials that are used temporarily. It has been recently reported that significant concerns about patient safety are caused by squamous cell carcinoma following cultured epidermal autografts. The functions of limbs are not recovered even after reconstruction and prostheses may be required to restore limited function. Deceased donors are a promising source of tissue for transplantation to restore shape and function. Skin allografts obtained from deceased donors may benefit burn patients and total limb allografts may help patients with mutilating trauma. Due to the need for aggressive immunosuppressive therapy to avoid graft rejection, skin or limb allografts are not commonly used. Immunosuppressive medications cause serious systemic effects, such as tumors, infections, and chronic problems, including renal failure, diabetes, and high blood pressure, although they can prolong allograft take [1–5]. Therefore, there is an urgent need to study non-pharmacologic methodologies to avoid allograft rejection.

Transplanted organs are exposed to a variety of cellular stresses, such as allograft rejection and injury induced by ischemia-reperfusion, which significantly elevates the level of apoptosis of allografts and may promote maladaptive scarring and healing. The release of apoptotic bodies which subsequently model the functions and phenotype of the recipient cells mediate apoptotic cells to transfer bioactive molecules. The innate and adaptive immune responses may be affected by apoptosis, which shifts the balance between rejection and tolerance of allogenic tissue. Accumulating evidence demonstrates that PIM2 is a common target for many anti-apoptotic pathways [6,7]. PIM1, PIM2, and PIM3 oncogenes are member of a threonine/serine kinase family. Studies have demonstrated that BAD on serine 112 is phosphorylated by PIM2, thereby achieving reversion of cell death induced by BAD [6].

Our understanding of the correlation between gene messenger RNAs (mRNAs) and human disease has changed since the discovery of microRNAs at the turn of the 21st century, which marked a new era in cell biology [7] and has been extended to those sequences in the residual 90% of eukaryotic genomes that produce non-coding RNAs [7]. MicroRNAs act as meta-controllers of gene expression and are pivotal in the cellular alterations required for development.

A recent study showed that dysregulation of PIM2 is functionally involved in the control of skin allograft survival in the treatment of severe burns by regulating cellular apoptosis [8]. By searching the online miRNA database (www.targetscan.org), PIM2 was identified as a target of miR-135-5p with the potential binding site within the 3'-UTR of the target gene, and the predicted binding site is highly conserved among species (predicted by *www.targetscan.org*). Therefore, we hypothesize that miR-135 might be a potent regulator of skin allograft survival via targeting PIM2. To test the hypothesis, we measured the expression of miR-135-5p and PIM2 using real-time PCR and Western blot in the skin samples collected from burn patients who received skin graft or in the fibroblast cells transfected with miR-135-5p mimics or inhibitors. The regulatory association between miR-135-5p and PIM2 was examined using bioinformatics analysis and luciferase assay.

Material and Methods

Patients and dermal sample collection

This study included 60 patients who were admitted to the Burn and Plastic Surgery Department, Jining Number 1 People's Hospital (Jining, Shandong, China) between Jan 2014 and Dec 2014. The patients were assessed at 3, 6, and 12 months after injury. Patients who met the following criteria were recruited to the study: patients with burned hands and/or wrists (deep partial skin-thickness burns) caused by contact with flame, steam, hot water, or other hot substances; For each patient, the total burned region should be less than 40% of the body surface area. On admission day, each patient was treated according to the standard treatment protocol. All burns were assessed for depth 24 h after admission. Skin samples were collected from skin with 2B° burn during surgery. The all those patients were divided into 2 groups based on the presence of rejection, with 30 patients in each group. The study protocol was approved by the Ethics Committee of Jining Number 1 People's Hospital. Written informed consent was obtained before the study.

RNA isolation and real-time PCR

Total RNA were extracted from dermal samples or culture cells using TRIZOL reagent (Invitrogen, Grand Island, NY). The extracts were then column-purified by using the RNeasy kit reagent (QIAGEN, Basel, Switzerland). RT-PCR was performed to assess the expression of miR-135-5p using specific primers using U6 snRNA as control. Expression of PIM2 was also analyzed with GAPDH as an internal control. PCR reactions were conducted on the MX3000P Real-time PCR system (StrataGen, Kirkland, WA) in accordance with the manufacturer's instructions. The resulting data were calculated using the $2^{-\Delta\Delta Ct}$ method. Final results are presented as ratios of miR-135-5p or PIM2 mRNA expression relative to that of the internal controls. Primer sequences of miR-135-5p

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were: Fw primer: 5'-AGCATAATACAGCAGGCACAGAC-3' and Rv primer: 5'-AAAGGTTGTTCTCCACTCTCTCAC-3'. PIM2: 5'-CGTAG CTAGTCGTCATACGTAGC-3' and Rv primer: 5'-CGTAGTACGTC GTATGTCGTAGTC-3'.

Cell culture and transfection

Fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Fibroblast cells (10^5/well) were seeded in 24-well plates on the night before transfection. On day of transfection, fibroblast cells were co-transfected with 100 nm wild-type or mutant-type vector and miR-135-5p mimics or scramble controls, or transfected with miR-135-5p mimics or negative controls alone using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations.

Vector construction and mutagenesis

Sequences of putative site of PIM2's 3'-UTR (untranslated region) that harbor the miR-135-5p were amplified through PCR with random primers and cloned into the XbaI site of a pGL3-Control vector (Promega, Madison, WI). Mutant constructs of the same miRNA seed sequences were generated using specific primers and the Site-Directed Mutagenesis reagent (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The mutation was verified by direct sequencing.

Cell proliferation assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the viability of transfected cells. In brief, after transfection, fibroblast cells (10^4/well) were prepared in a 96-well plate and treated with 50 ml MTT solution (0.1 mg/ml) and incubated at 37°C. After 4 h, 150 ml dimethylsulfoxide was added to each well and incubated at room temperature for 15 min. Finally, Optical density of cells was read at a wavelength of 580 nm at 1 h, 2 h, 4 h, and 8 h.

Luciferase assay (3'UTR miRNA)

Fibroblast cells were cotransfected with 100 nm wild-type or mutant-type vector and miR-135-5p mimics or scramble controls on the lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously. At 48 h after transfection, the transfected cells were subjected to the Dual-Luciferase Reporter Assay Luciferase activity of transfected cells was determined using a luminometer and a dual-luciferase reporter system (Promega, Madison, WI). Renilla luciferase plasmid was also transfected to cells as an internal control. Each experiment was conducted with 3 independent specimens and repeated twice. All resulting values are presented as fold difference of firefly luciferase activity of miR-135-5p transfected cells relative to luciferase activity of Renilla luciferase plasmid transfected cells. Each experiment was conducted 2 times, with consistent results.

Western blot analysis

Western blot analysis was performed to determine the expression level of PIM2 in sampled tissues and the transfected fibroblast cells. In brief, total protein of sample tissues or fibroblast cells were extracted using RIPA buffer. The protein concentrations of extracts were measured through Bradford method (Bio-Rad Laboratories, Hercules, CA). We loaded 10-mg of total protein on 15% SDS-PAGE. After electrophoresis, protein blots were transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA). The membranes were then blocked with non-fat powdered milk and probed with specific primary antibodies (anti-PIM2 antibody, Abcam, Boston, MA, 1:2000, overnight, 4C; anti-β-actin antibody, Abcam, Boston, MA, 1:15000, RT, 2 h), and then washed with PBS and probed with secondary antibody (Abcam, Boston, MA, 1:20000, 1 h, RT). Finally, the conjugated protein blots were visualized using ECL Chemiluminescence reagent (GE Healthcare Bio-Sciences, Pittsburgh, PA) and IMAGEJ software was used to quantify each band. The expression of β-actin was also determined and used as internal control. All results are presented as fold differences relative to internal control.

Apoptosis analysis

Annexin V-FITC staining and flow cytometry were used to assess the effect of PIM2 or miR-135-5p on cell apoptosis. Briefly, 24 h after transfection, the cells were harvested through centrifugation and fixed with 70% ethanol, then stained with 5 µl Annexin V-FITC and 10 µl propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) for 20 min in the dark. Finally, the pellet was resuspended in PBS and the stained cells were counted using as flow cytometer (BD FACSCalibur System, Becton Dickinson (Pty) Ltd., CA).

Statistical analysis

All data values are presented as mean ±SEM of more than 3 independent experiments. Comparisons among more than 3 groups were performed with 1-way, 2-way, or 3-way ANOVA without repeated measures, as appropriate. Comparisons among groups (less than 3 groups) performed with 1-way ANOVA followed by Dunnett's post-hoc test. Comparisons among ≥3 groups performed with 2- or 3-way ANOVA followed by stratified independent *t* test with Bonferroni corrections for multiple comparisons. Statistical significance was defined as $p \leq 0.05$. All values from each experiment are expressed as mean ±SD. All data analyses were conducted on SPSS19.0 (IBM, Chicago, IL).

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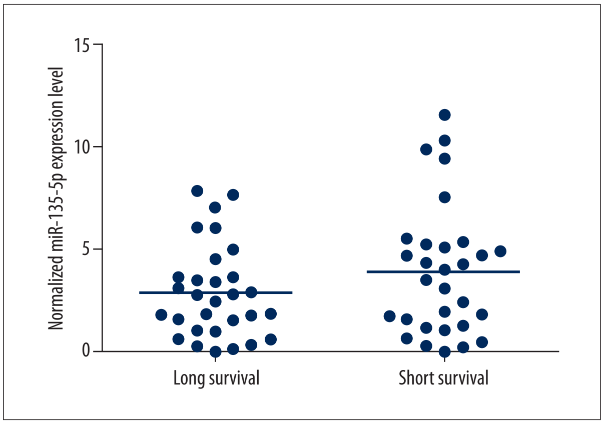

Figure 1. The expression level of miR-135-5p was higher in the short survival group than in the long survival group.

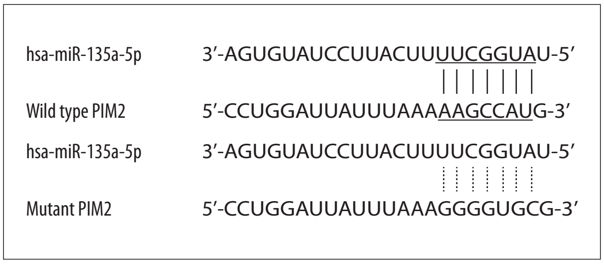

Figure 2. PIM2 as the candidate target gene of miR-135-5p in cells with the 'seed sequence' in the 3'UTR.

Results

MiR-135-5p is involved with the prognosis of burn patients after skin transplantation

We collected data on burn patient after skin transplantation; these patients were followed up and we then divided them into 2 groups based on the presence of rejection. Using real-time PCR, we found that the expression level of miR-135-5p was higher in the short survival group. The results indicate that miR-135-5p is negatively related to the survival of the graft (Figure 1).

PIM2 was a virtual target of miR-135-5p

It has been reported that miR-135-5p is involved with many diseases. In this study we aimed to understanding the relationship between the miR-135-5p level and burn patient survival after skin transplantation. We used online miRNA target prediction tools to search the regulatory gene of miR-135-5p, and consequently identified PIM2 as the candidate target gene of miR-135-5p in cells with the 'seed sequence' in the 3'UTR (Figure 2). Furthermore, to validate the regulatory relationship between miR-135-5p and PIM2, we also conducted luciferase activity reporter assay in cells, showing that only the luciferase activity from the cells cotransfected with miR-135-5p and wild-type PIM2 3'UTR decreased significantly (Figure 2), while cells

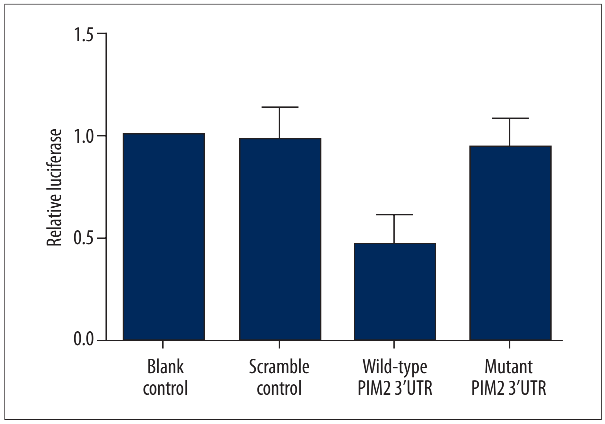

Figure 3. Luciferase activity reporter assay was conducted to verify PIM2 as the direct target gene of miR-135-5p.

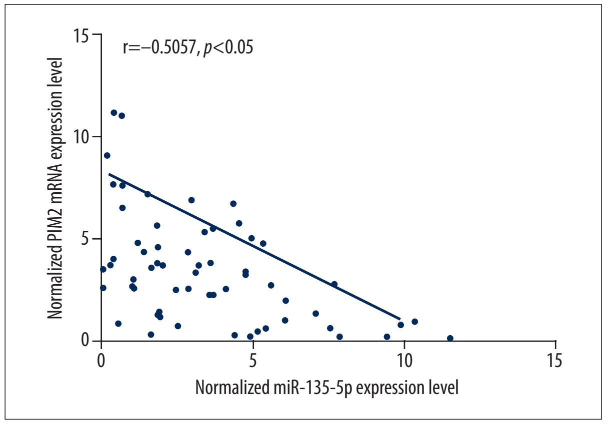

Figure 4. The correlation between the expression level of miR-135-5p and PIM2 mRNA between long survival and short survival groups (long survival n=30 and short survival n=30).

cotransfected with miR-135-5p and mutant PIM2 3'UTR were comparable to scramble control (Figure 3). The results confirmed that PIM2 is a validated target of miR-135-5p in cells. To further define the modulatory relationship between miR-135-5p and PIM2, we then analyzed the correlation between the expression level of miR-135-5p and PIM2 mRNA among the tissues (n=60), showing a negative regulatory relationship (Figure 4).

Determination of expression patterns of miR-135-5p and PIM2 in tissues with different groups

The tissues of 2 different groups (long survival n=30 and short survival n=30) were used to further explore the impacts on the interaction between miR-135-5p and PIM2 3'UTR. Using real-time PCR, we found the expression of PIM2 mRNA (Figure 5A) decreased in the short survival group compared with the long survival group. The expression of PIM2 protein (Figure 5B) was measured by densitometry analysis, and we found it decreased in the short survival group compared with the long

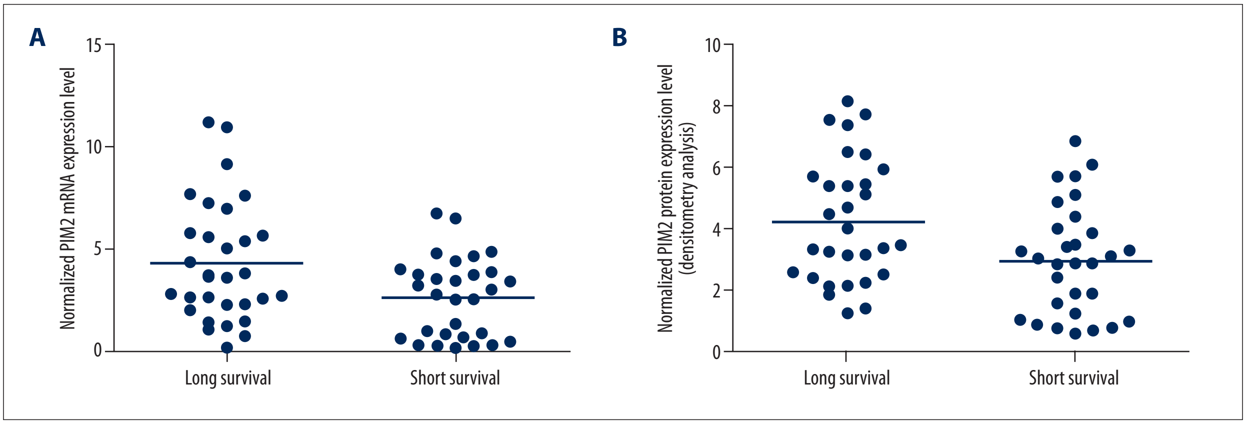

Figure 5. The expression of PIM2 mRNA (**A**) and protein (**B**) was decreased in the short survival group compared with the long survival group.

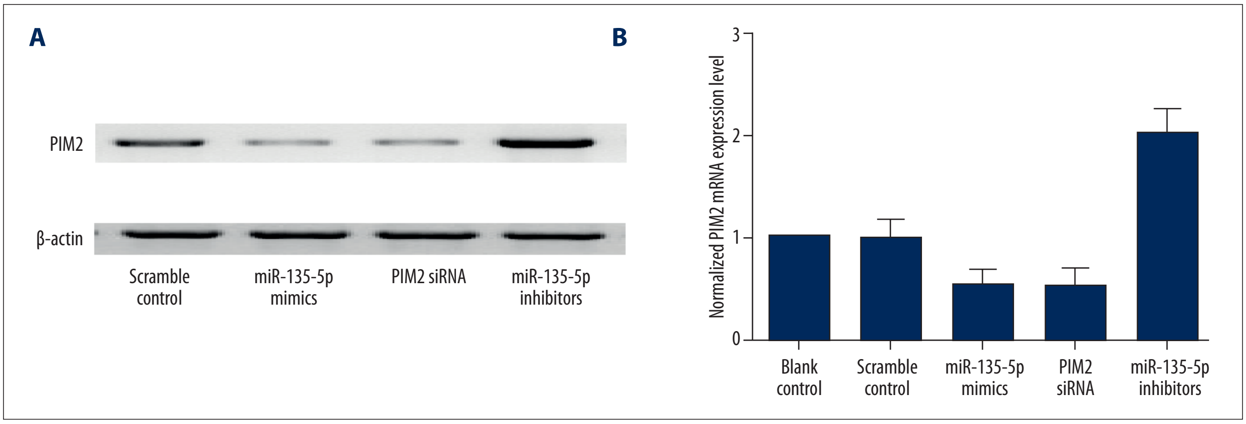

Figure 6. When transfected with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors, the expression level of PIM2 protein (**A**) and mRNA (**B**) treated with miR-135-5p mimics and PIM2 siRNA decreased, while cells treated miR-135-5p inhibitors increased.

survival group. To further validate the hypothesis of the negative regulatory relationship between miR-135-5p and PIM2, we transfected the cells with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors. As shown in Figure 6, the protein (upper panel) and mRNA (lower panel) expression level of PIM2 of cells treated with miR-135-5p mimics and PIM2 siRNA were apparently lower than the scramble control, while cells treated miR-135-5p inhibitors were apparently higher than the scramble control, validating the negative regulatory relationship between miR-135-5p and PIM2.

miR-135-5p and PIM2 interfered with the viability in fibroblast cells

We also investigated the relative viability of cells when transfected with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors. Cells transfected with miR-135-5p inhibitors showed clearly downregulated viability (Figure 7A) when compared with the scramble controls, while cells transfected with miR-135-5p mimics and PIM2 siRNA showed comparably higher viability, indicating that miR-135-5p reduced the viability of cells and PIM2 reduced the viability of cells.

miR-135-5p and PIM2 interfered with the apoptosis in fibroblast cells

We then investigated the relative apoptosis of cells when transfected with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors. When transfected with miR-135-5p mimics and PIM2 siRNA, there were more surviving cells and fewer apoptotic cells were than in the scramble controls, while cells transfected with miR-135-5p inhibitors showed comparably fewer surviving cells and more apoptotic cells. The results indicated miR-135-5p inhibited apoptosis and PIM2 accelerated apoptosis (Figure 7B).

Discussion

In this study, the expression level of miR-135-5p was determined in 60 tissue samples divided into 2 groups based on the

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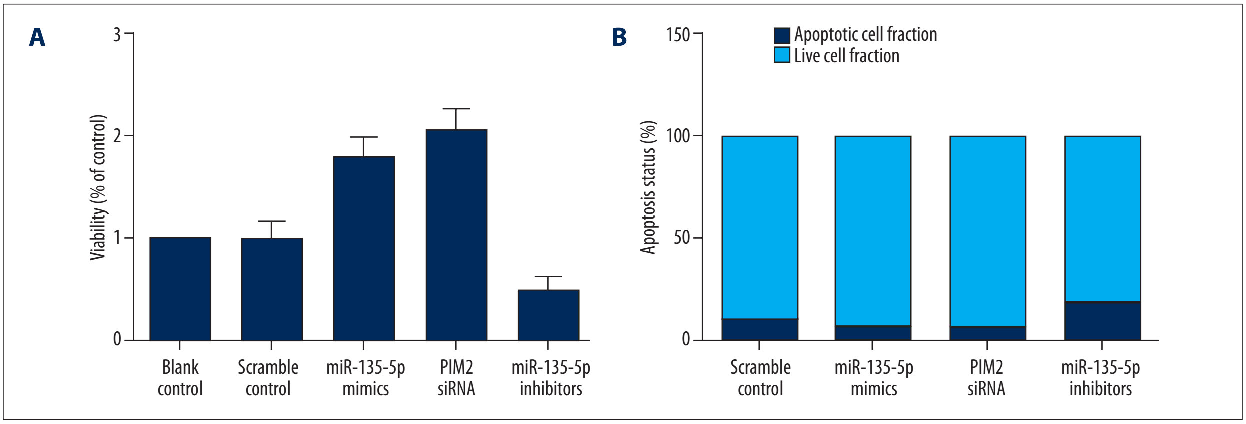

Figure 7. (**A,B**) Cells transfected with miR-135-5p inhibitors showed clearly downregulated viability, while cells transfected with miR-135-5p mimics and PIM2 siRNA showed higher viability. Cells transfected with miR-135-5p mimics and MP2 siRNA had inhibited apoptosis while cells transfected with miR-135-5pinhibitors had accelerated apoptosis.

presence of rejection (long survival n=30 and short survival n=30). We found that miR-135-5p was substantially downregulated in the long survival group. We then searched the miRNA database online with the "seed sequence" located within the 3'-UTR of the target gene, and then validated PIM2 to be the direct gene via luciferase reporter assay system. We also established the negative regulatory relationship between miR-135-5p and PIM2 via studying the relative luciferase activity.

PIM is integral to the composition of the mycobacterial envelope and is found in various phosphatidyl-myo-inositol mannosides (PIM) 1–6. PIM2 has many biological functions [9–16]. It was demonstrated that PIM2 fostered active macrophages mediated by TLR2, which led to activation of mitogen-activated protein kinases (MAPK), AP-1, and nuclear factor-κB (NF-κB) [17]. PIM2 also recruited NKT cells into granulomas and has pulmonary granuloma-forming properties [18]. Furthermore, it was suggested that PIM induces adherence of *M. tuberculosis* bacilli to cells that are nonphagocytic [13]. Therefore, an inflammatory responses similar to mycobacteria bacilli could be initiated or affected by PIM2, the mycobacterial envelope antigen.

Recent studies have emphasized that the apoptosis-associated intercellular communication networks play a crucial role in the modulation of immune responses and allograft remodeling in transplantation. Transplanted organs must respond to a variety of metabolic and immunologic stressors that increase the proportion of dying cells, and during rejection apoptosis is strictly orchestrated and peaks in the activation of caspases. Serine/threonine kinases, consisting of PIM1, PIM2 and PIM3, are encoded by the PIM family which represents a small family of proto-oncogenes and is very important in tumorigenesis [19]. The PIM kinases trigger various effects in diverse tumors [20–22]. It has been shown that 5-(3-trifluoromethylbenzylidene) thiazolidine-2,4- dione is a specific suppressor of the PIM kinases, which might lower the capacity of PIM to trigger G1 phase cell cycle arrest and apoptosis, and phosphorylate the BAD BH3 protein [23–25]. In this study, we conducted real-time PCR and Western blot analysis to study the mRNA and protein expression level of PIM2 among the 2 different groups (long survival n=30 short survival n=30) or cells treated with scramble control, miR-135-5p mimics, PIM2 siRNA, and miR-135-5p inhibitors, indicating the negative regulatory relationship between MiR-135-5p and PIM2. We also conducted experiments to investigate the influence of miR-135-5p and PIM2 on viability and apoptosis of cells. Our results showed miR-135-5p reduced the viability of cells, while PIM2 negatively interfered with the viability of cells, miR-135-5p inhibited apoptosis, and PIM2 accelerated apoptosis.

Fibroblast cells are crucial in alloimmune responses. By direct or indirect recognition of allogeneic antigen, these cells can be activated and trigger delayed hypersensitivity reactions to interrupt the allograft [26]. Moreover, diverse subsets can be obtained via activation of fibroblast cells, which can inhibit the function or activation of traditional CD4+CD25- T effector cells to inhibit allograft rejection [27]. Furthermore, there is a correlation between the intracellular expression of the transcriptional factor known as Foxp3 and the expression of CD25 (the α-chain of the IL-2 receptor) in mice, which suppresses IL-2 gene transcription [28]. It has been shown that fibroblast cells, which are a donor antigen, can enhance donor-specific tolerance and modulate allo-responses in a skin transplantation model [29]. Intriguingly, Foxp3 triggers the expression of PIM2 in fibroblast cells, resulting in Foxp3-expressing fibroblast cells in the presence of rapamycin [30]. The studies demonstrate that there may be a correlation between PIM2 and the function of fibroblast cells in allograft rejection [31]. The activation of target of rapamycin (TOR) and the effector enzyme Akt in the phosphatidylinositol 3-kinase pathway partly mediate the growth and survival of fibroblast cells. TOR is a crucial trigger of signal transduction, which is dependent on Akt [32]. The inhibition of mTOR mediated by rapamycin

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impedes crucial effector functions, including cytokine production and migration, and results in limitation of fibroblast expansion [6]. PIM2 and Akt share many typical downstream targets, including BAD and 4E-binding protein-1 [33–35]. It has been demonstrated that the expression of PIM2 is necessary for compensation of TOR suppression by rapamycin in fibroblast cells, demonstrating that PIM2 kinase might be a substitutive pathway for fibroblast survival [36].

Conclusions

MiR-135-5p is involved with the prognosis of burn patients after skin transplantation. PIM2 is a virtual target of miR-135-5p, and there is a negative regulatory relationship between miR-135-5p and PIM2. MiR-135-5p and PIM2 interfered with the viability and apoptosis of cells.

Conflict of interest

None.

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