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MicroRNA 26a prolongs skin allograft survival and promotes regulatory T cell expansion in mice

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Conflicts of interest
The authors declare no conflicts of interest.

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Summary

MicroRNA 26a (Mir-26a) has been reported to play functions in cellular differentiation, cell growth, cell apoptosis, and metastasis. However, the role of Mir-26a in transplant rejection has never been investigated. Full-thickness skin grafts 1–2 cm in diameter were obtained from the tail-skin CBA/J donor mice and transplanted onto the back of wild-type C57Bl/6 recipient mice. Vectors encoding pre-Mir-26a (LV-26a) and an empty lentiviral vector (LV-Con) delivered approximately 2×10^7 transforming units of recombinant lentivirus were injected to mice once through the tail vein. Mir-26a overexpression results in prolonged skin allograft survival (MST = 9.5 days in LV-Con mice; MST = 22 days in LV-26a mice. $P < 0.01$) and promoted regulatory T cells (Tregs) expansion. The prolonged skin allograft survival induced by LV-26a was abrogated by depletion of Tregs with anti-CD25 antibodies. Mir-26a significantly promoted IL-10 expression and suppressed the expression of IL-6, IL-17, and IFN-γ. Furthermore, IL-6 overexpression led to complete suppression of the Mir-26a-induced upregulation of Foxp3. The prolonged allograft survival induced by LV-Mir-26a was also completely abrogated by IL-6 overexpression. In conclusion, Mir-26a prolongs skin allograft survival and promotes Tregs expansion in part through inhibition of IL-6 expression.

Introduction

Transplantation remains the best treatment option to correct certain types of organ failure and tissue damage (i.e. kidney, heart, lung, etc.). However, the long-term use of globally immunosuppressive drugs to prevent rejection carries with it some serious risks, including infection and cancer due to their lack of target specificity, as well as graft loss due to their toxicity [1]. Thus, identifying safe methodologies to induce donor-specific allograft survival is a top priority.

MicroRNAs (miRNAs) are endogenously expressed, small noncoding RNAs that negatively regulate gene expression by causing degradation of target mRNAs, inhibition of the translation of these mRNAs or both [2]. miRNAs take a part in crucial cellular processes such as the stress response, development, differentiation, apoptosis, and proliferation [3]. Mir-26a has been reported to play functions in cellular differentiation, cell growth, cell apoptosis, and metastasis [4–7]. Recent study indicated that Mir-26a modulated Th17/Treg balance and attenuated multiple sclerosis in a mouse experimental autoimmune encephalomyelitis (EAE) model [8]. However, the role of Mir-26a in the skin allograft rejection has never been investigated.

In our current study, we demonstrate a crucial role for Mir-26a in modulating Tregs expansion and prolonging skin allograft survival. Mir-26a overexpression results in prolonged skin allograft survival and promoted Tregs expansion. The prolonged skin allograft survival induced by LV-26a was abrogated by depletion of Tregs with anti-CD25 antibodies. Mir-26a significantly promoted IL-10 expression and suppressed the expression of IL-6, IL-17, and IFN-γ. Furthermore, IL-6 overexpression led to complete suppression of the Mir-26a-induced upregulation of Foxp3.

Materials and methods

Animals

CBA/J (H-2^k) and C57Bl/6 (H-2^b) mice were obtained from Henan University of Science and Technology. IL-10 knockout C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were used at 8–10 weeks of age. All protocols were approved by the institutional ethics committee on animal use of the Henan University of Science and Technology.

Skin transplantation

Full-thickness skin grafts 1–2 cm in diameter were obtained from the tail-skin CBA/J donor mice and transplanted onto the back of wild-type C57Bl/6 recipient mice or IL-10 knockout C57Bl/6 mice [9]. Graft rejection was defined as the first day on which the entire graft was necrotic. To deplete Tregs, anti-CD25 antibodies (PC61; BioXCell, West Lebanon, NH, USA) were administered to mice at a dose of 0.3 mg/mouse on 2 consecutive days from 1 day before skin transplantation [10].

Vector construction and lentivirus production

A 200-bp DNA fragment corresponding to pre-Mir-26a and its flanking sequences were amplified from mouse genomic DNA and were subsequently cloned into pLVTHM lentiviral vector (http://www.addgene.org/Didier_Trono) [8]. The production, purification, and titration of lentivirus were performed as described by Tiscornia and colleagues [11]. The packaged lentiviruses were named LV-26a and LV-IL-6. The empty (untransformed) lentiviral vector LV-Con served as control.

Protocols of LV-26a administration

Vectors encoding pre-Mir-26a (LV-26a) and an empty lentiviral vector (LV-Con) delivered approximately 2×10^7 or 5×10^7 transforming units of recombinant lentivirus were injected to mice once through the tail vein; the efficacy of lentivirus infection was assessed 7 days later by quantitative real-time PCR analysis of Mir-26a expression in the skin of LV-26a-infected mice and LV-Con-infected mice. Lentivirus-infected mice were established skin transplantation model on day 7 after virus injection.

Histology

The histological analysis of the skin graft was performed by staining 5-mm sections of paraffin-embedded tissues with hematoxylin and eosin (HE).

Real-time PCR

Skin and draining lymph node samples were initially snap-frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent and the miRNeasy Mini kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. cDNA was prepared as recommended (Progema) and used as the template for quantitative PCR. Levels of Mir-26a and mRNA for IL-6, IL-10, IL-17, Foxp3, IFN-γ, TGF-β, and HPRT were analyzed by real-time PCR, performed according to the manufacture's instruction (Takara BioInc., Otsu, Shiga, Japan). The forward RT-PCR primer for Mir-26a was 5′-AAGGAGAACCCGTAGATCCG-3′, and the reverse one was 5′-GTGCAGGGTCCGAGGTATTC-3′. Transcript levels were normalized to the expression of HPRT. Analyses were performed with the Sequence Detection Software 1.9 (SDS, Beijing, China).

FACS analyses

For the detection of $CD4^+Foxp3^+$Tregs, cells obtained from the axillary lymph node were resuspended in FACS buffer (PBS with 2% FBS) and stained for flow cytometry analysis. To block Fc receptor-mediated binding of antibodies, mononuclear leukocytes were resuspended in FACS buffer with hamster anti-mouse CD16/32, clone 2.4G2 (Fc BlockTM; BD Bioscience, Houston, TX, USA) for 20 min. These cells were then washed, placed on ice for 30 min, and stained with fluorochrome-conjugated antibodies. Cells were washed twice in buffer and reserved for analysis. Intracellular staining for Foxp3 was performed on lymph node cells according to the manufacturer's procedure (eBioscience, San Diego, CA, USA).

Western blotting

The protein levels were determined by Western blotting. Protein extracted from tissue was separated on 10% SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes (Pierce, Rockford, IL, USA). After being blocked with 5% nonfat milk in TBS for 3 h, the membranes were incubated with indicated primary antibodies (0.2 μg/ml) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody (1:5000) for 3 h. β-actin was used as a loading control for comparison between samples.

Nucleofection

Nucleofection was performed with Mouse T Cell Nucleofector® Kit and Nucleofector device (Amaxa, Koelin, Germany). First, 1×10^7 naive $CD4^+$ T cells were resuspended in 100 μl nucleofector® solution. 2.5 μg pmaxGFP® Vector or 100 pmol oligonucleotides (including pre-Mir-26a,

pre-Mir-ctrl, anti-Mir-26a, and anti-Mir-ctrl) were added into the solution and mixed gently. Then the mixtures were gently transferred to electroporation cuvettes and placed in the Nucleofector device. Cells were nucleofected in the X-01 program. Finally, transfected cells were transferred to a 12-well plate with 1.5 ml prepared Mouse T Cell Nucleofector® Medium in each plate and incubated in a humidified 37 °C/5% CO_2 incubator until analysis. The transfection efficiency, which was detected by monitoring green fluorescent protein (GFP) expression under fluorescence microscope 8 h after transfection, is about 55%.

$CD4^+$ T cell activation and polarization

Four hours after nucleofection, $CD4^+$ T cells were activated by 5 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28. For propagation under Treg condition, 100 μg/ml rIL-2, 10 ng/ml rTGF-b1, 10 mg/ml anti-IFN-γ, and 10 mg/ml anti-IL-4 were provided. All antibodies used were purchased from eBioscience. All cytokines used were purchased from Peprotech (Rocky Hill, NJ, USA).

MiRNA target validation

A 328-bp fragment of the IL6 3′ untranslated region (UTR) was amplified by PCR and cloned into the pGLO vector (Promega Corp., Madison, WI, USA), downstream of the firefly luciferase gene. This vector was named wild-type (wt) 3′ UTR. Site-directed mutagenesis of the Mir-26a-binding site in IL-6 3′ UTR was performed using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen, Grand Island, NY, USA) and named mutant (mt) 3′ UTR. For reporter assays, wt or mt 30 UTR vector and the control vector pRL-TK (Promega) were cotransduced in HEK293 cells. Luciferase activity was measured 36 h after transduction, using the Dual-luciferase reporter assay system [8].

Statistics

Data are presented as means ± SD. Differences were evaluated using unpaired Student's *t*-test between two groups and one-way ANOVA for multiple comparisons, followed by a post hoc Student–Newmann–Keuls test when necessary. Survival curves were estimated by the Kaplan–Meier method and compared with a log-rank test. All analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA), and statistical significance was set at $P < 0.05$.

Results

Mir-26a prolongs allogeneic skin graft survival

We first explored the expression of Mir-26a in skin allograft by quantitative reverse transcriptase PCR. Skin allograft at day 3 after transplantation showed significantly lower expression of Mir-26a (Fig. 1a). Next, we investigated the potential effects of Mir-26a on skin transplantation through the construction of lentiviral vectors. Vectors encoding pre-Mir-26a (LV-26a) and an empty lentiviral vector (LV-Con) delivered approximately 2×10^7 or 5×10^7 transforming units of recombinant lentivirus to mice by injection through the tail vein. The efficacy of lentivirus infection was assessed 7 days later by quantitative real-time PCR analysis of Mir-26a expression in the skin of LV-26a-infected mice and LV-Con-infected mice ($P < 0.01$, Fig. 1b). Lentivirus-infected mice were established skin transplantation on day 7 after virus injection. Relative to LV-Con-infected mice, LV-26a (2×10^7)-infected mice exhibited significantly prolonged allograft survival (MST = 9.5 days in LV-Con mice; MST = 22 days in LV-26a mice ($P < 0.01$, Fig. 1c). Consistent with prolonged skin allograft survival, LV-26a (2×10^7)-infected mice presented healthier skin morphology with fewer infiltrated mononuclear cells on day 3 after skin transplantation (Fig. 1d). Relative to LV-26a (2×10^7)-infected mice, LV-26a (5×10^7)-infected mice showed prolonged allograft survival but with no significant difference (Fig. 1c). This indicated that higher dose injection cannot further prolong allograft survival. So, in our further experiment, the LV-26a group indicated the mice injected with 2×10^7 transforming units of recombinant lentivirus. Next, we investigated the Mir-26a expression in T lymphocytes from draining lymph node on day 3 after transplantation and found that the Mir-26a expression in T lymphocytes was significantly increased in LV-26a group when compared with LV-Con group (Fig. 1e).

Mir-26a alters cytokine expression

To closely examine the expression of molecules related to allograft tolerance and rejection, we harvested draining lymph nodes and skin grafts early (day 3) and preceding allograft rejection (day 10) post-transplantation. Interestingly, Foxp3 expression was higher in the draining lymph nodes and skin allograft of LV-26a-infected mice than control mice on days 3 and 10 after transplantation. We also observed lower expression of IL-6, IL-17, and IFN-γ in the draining lymph nodes and skin allograft of LV-26a-infected mice when compared with control mice on days 3 and 10 after transplantation. The expression of IL-10 was higher in the draining lymph nodes of LV-26a-infected mice than control mice on days 3 and 10 after transplantation, and this increase was only displayed on day 10 after transplantation in skin allograft. There was no significant difference in the expression of TGF-β in skin allograft or draining lymph node (Fig. 2a and b).

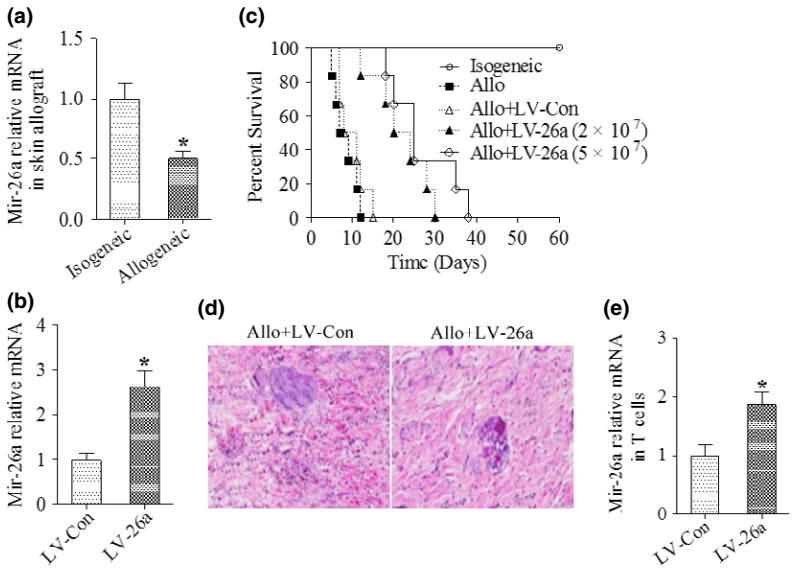

Figure 1 Mir-26a prolongs allogeneic skin graft survival. (a) Mir-26a mRNA expression was analyzed by RT-PCR method; (b) quantitative PCR analysis of Mir-26a mRNA expression in the skin of mice infected with LV-Mir-26a or LV-Con (day 7 after lentivirus administration); (c) indicating skin allograft survival; (d) histological analysis of skin rejection on day 3 after transplantation. (e) Mir-26a mRNA expression in T lymphocytes was analyzed by RT-PCR method. *$P < 0.01$, $n = 6$.

Mir-26a promotes regulatory T cell expansion *in vivo* and *in vitro*

First, we measured Tregs after skin transplantation *in vivo*. LV-26a induced significant expression of $CD4^+Foxp3^+$ Tregs in draining lymph node on day 3 and day 10 after skin transplantation (Fig. 3a). We also detected the Foxp3 expression in skin allograft on days 3 and 10 after transplantation, and the mRNA and protein levels of Foxp3 were both significantly increased in LV-26a group when

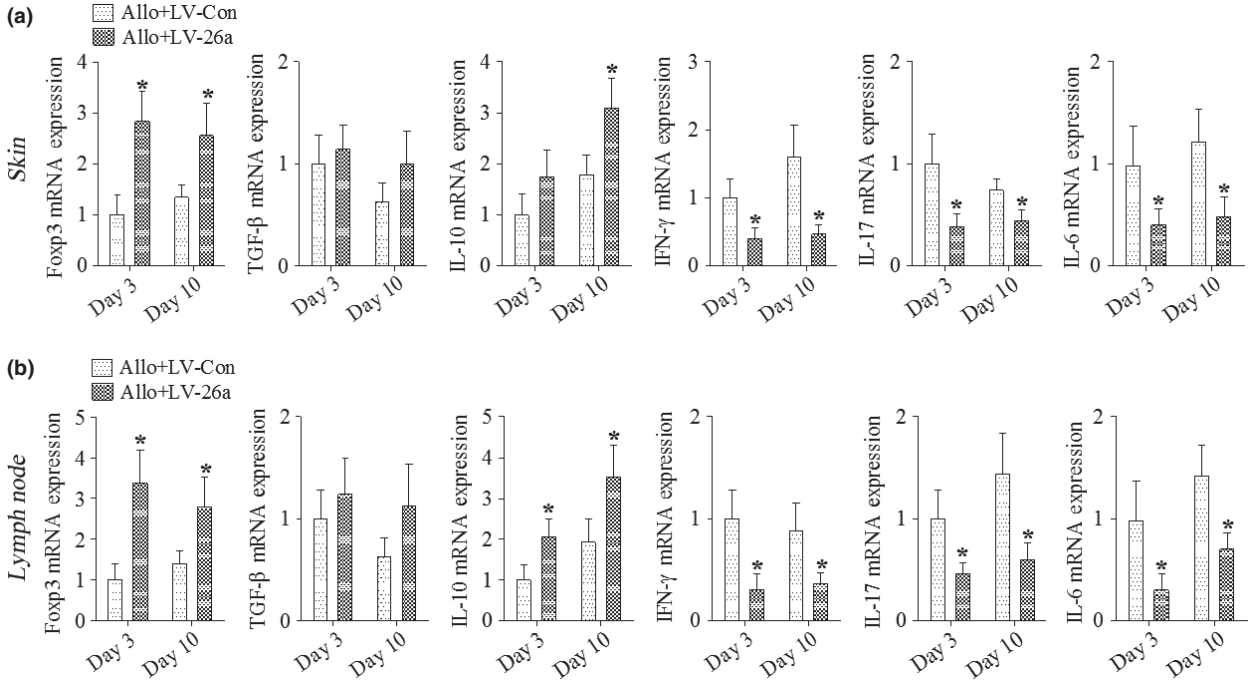

Figure 2 Mir-26a alters cytokine expression. Tissues were analyzed on days 3 and 10 after transplantation. RNA was isolated from (a) skin and (b) draining axillary lymph nodes. Gene expression of Foxp3, TGF-β, IL-10, IFN-γ, IL-17, and IL-6 was assessed by quantitative RT-PCR. *$P < 0.01$, $n = 6$.

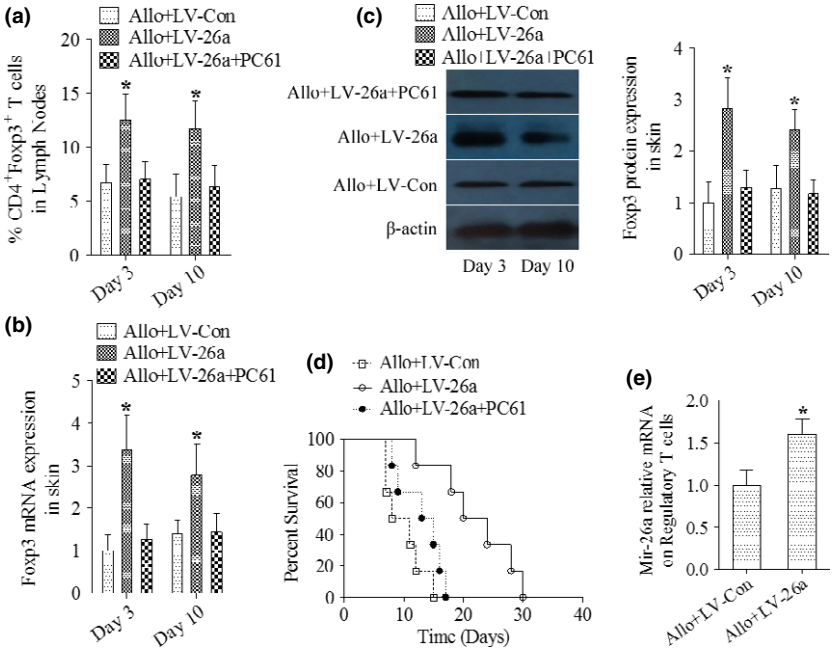

Figure 3 Mir-26a modulates regulatory T cell expansion *in vivo*. (a) The proportions of $CD4^+Foxp3^+$ Tregs among $CD4^+$ T cells in draining lymph nodes were analyzed by FACS; (b) Foxp3 mRNA expression in skin allograft was analyzed by Q-PCR; (c) Foxp3 protein expression in skin allograft was analyzed by Western blot; (d) indicating skin allograft survival. (e) Mir-26a mRNA expression on $CD4^+Foxp3^+$ Tregs was analyzed by RT-PCR method. $CD4^+Foxp3^+$ Tregs in draining lymph node were isolated by magnetic bead. $*P < 0.01$, $n = 6$.

compared with LV-Con group (Fig. 3b and c). When anti-CD25 antibodies (PC61) were administrated, expansion of Tregs in both skin allograft and draining lymph nodes was completely abrogated (Fig. 3a–c). The prolonged allograft survival induced by LV-26a was also reversed by additional administration of anti-CD25 antibodies (Fig. 3d). In our previous results, we showed that the Mir-26a expression in T lymphocytes was significantly increased after LV-26a infection. Next, we isolated $CD4^+Foxp3^+$ Tregs from draining lymph node on day 3 after transplantation and found that the Mir-26a expression on $CD4^+Foxp3^+$ Tregs was also markedly increased in LV-26a group when compared with LV-Con group (Fig. 3e).

To explore the roles of Mir-26a in Tregs differentiation *in vitro*, Mir-26a was overexpressed and inhibited by pre-Mir-26a and anti-Mir-26a, respectively. The results showed that the frequency of Tregs was higher in $CD4^+$ T cells which were transfected with pre-Mir-26a than in those which were transfected with pre-Mir-ctrl and anti-Mir-26a (Fig. 4a). In contrast, it was lower in $CD4^+$ T cells which were transfected with anti-Mir-26a than in those which were transfected with anti-Mir-ctrl (Fig. 4a). Similarly, Foxp3 expression was increased in pre-Mir-26a groups compared with those in pre-Mir-ctrl groups and anti-Mir-26a groups and was decreased in anti-Mir-26a groups compared with those in anti-Mir-ctrl groups (Fig. 4b).

Mir-26a promotes Tregs expansion through repression of IL-6 expression

A previous study reported that Tregs from IL-10 knockout mice have defects in immunoregulation. To assess the role of IL-10 in Mir-26a-mediated allograft protection, we administered LV-26a in IL-10 knockout mice. The Foxp3 mRNA expression was lower in LV-26a-infected IL-10 deficiency mice when compared with LV-26a-infected wild-type mice, and LV-26a also induced expansion of Tregs in IL-10 knockout mice as well as wild-type mice (Fig. 5a and b). The skin allograft survival in LV-26a-infected wild-type mice was longer than LV-26a-infected IL-10 knockout mice (MST = 22 days in WT/LV-26a group, MST = 14.5 days in IL-$10^{-/-}$/LV-26a group, $P < 0.05$, Fig. 5c), and LV-26a also prolonged skin allograft survival in IL-10 knockout mice as well as wild-type mice (MST = 14.5 days in IL-$10^{-/-}$/LV-26a group, MST = 6 days in IL-$10^{-/-}$/LV-Con group, $P < 0.01$, Fig. 5c). These data suggested that IL-10 is dispensable in the LV-26a-mediated expansion of Tregs and protective effects for skin allograft.

TGF-β induces Foxp3 expression and promotes Tregs function [12]. It also works together with IL-6 to induce Th17 differentiation [13]. So we investigated the expression of cytokine IL-6 and TGF-β *in vitro*. Results showed that pre-Mir-26a significantly decreased IL-6 expression, but

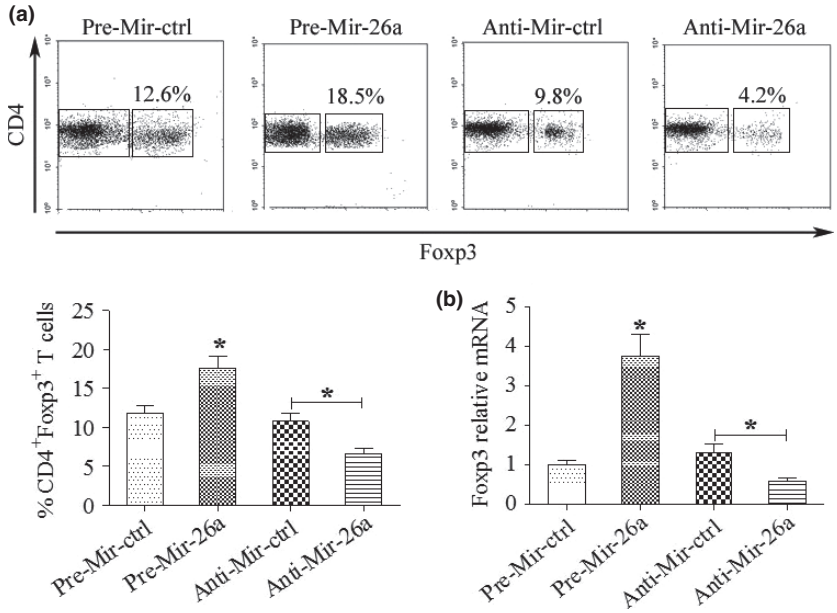

Figure 4 Mir-26a promotes regulatory T cell expansion *in vitro*. Pre-Mir-ctrl, pre-Mir-26a, anti-Mir-ctrl, and anti-Mir-26a were transfected into $CD4^+$ T cells, which were then activated and polarized. (a) The frequencies of Tregs in $CD4^+$ T cells were determined by flow cytometry 4 days later. Tregs were gated with $CD4^+Foxp3^+$. Representative FACS pictures from a single case are shown. And the percentages of positive cells in $CD4^+$ T cells are shown in each panel. The collective results of three independent experiments are shown in the histograms as mean ± SD. (b) The Foxp3 mRNA levels were analyzed by Q-PCR 3 days after transfection and activation. $*P < 0.01$, $n = 6$.

have no effect on TGF-β expression (Fig. 6a). To further investigate the role of IL-6 in the promoted Tregs expansion induced by LV-26a, mice were coinfected with LV-26a and LV-IL-6 encoding the full-length IL-6 coding sequence but without the 3′ UTR. Results showed the increased expansion of $CD4^+Foxp3^+$ Tregs in draining lymph nodes, and skin allograft induced by LV-Mir-26a was completely abrogated by IL-6 overexpression (Fig. 6b and c). The prolonged allograft survival induced by LV-Mir-26a was also completely abrogated by IL-6 overexpression (MST = 22 days in LV-26a group, MST = 11 days in LV-26a + LV-IL-6 group, $P < 0.01$, Fig. 6d).

To support our data suggesting that the Mir-26a level was inversely associated with IL-6, a luciferase reporter assay was performed to determine whether Mir-26a could directly target the 3′ UTR of IL-6 mRNA in HEk293 cells. The results showed that Mir-26a significantly decreased the luciferase reporter activity of wild-type 3′ UTR but not mutant 3′ UTR of IL-6 (Fig. 6e).

Discussion

Our current study provides strong evidence that Mir-26a is involved directly in skin allograft rejection by modulating Tregs expansion. Mir-26a overexpression results in prolonged skin allograft survival and promoted Tregs expansion. The prolonged skin allograft survival induced by LV-26a was abrogated by depletion of Tregs with anti-CD25 antibodies. Mir-26a significantly promoted IL-10 expression and suppressed the expression of IL-6, IL-17, and IFN-γ. Furthermore, IL-6 overexpression led to complete suppression of the Mir-26a-induced upregulation of Foxp3. These findings establish an immune regulatory role for Mir-26a *in vivo* in a relevant clinical disease model and provide the rationale for selectively targeting this signaling pathway as a novel therapeutic strategy to ameliorate transplant rejection.

Tregs are of great interest for immunotherapy as they could be used to prevent transplant rejection and in the treatment of autoimmune diseases. Adoptive transfer of $CD4^+CD25^+$ Tregs has been successfully used in experimental immunosuppression in several animal models [14–16]. The role of Tregs in establishing and maintaining immune tolerance is increasingly appreciated [17–19]. The actions of Tregs can be mediated by the production of anti-inflammatory cytokines such as IL-10 or TGF-β, by CTLA-4-mediated inhibition or direct cell–cell contact [20,21]. In our current study, Mir-26a promoted Tregs expansion, suppressed expression of IL-6, IL-17, and IFN-γ, and prolonged skin allograft survival. The prolonged allograft survival after skin transplantation induced by LV-26a was abrogated by depletion of Tregs with anti-CD25 antibodies. These results suggested that expanded Tregs contributed to prolonged skin allograft survival induced by Mir-26a

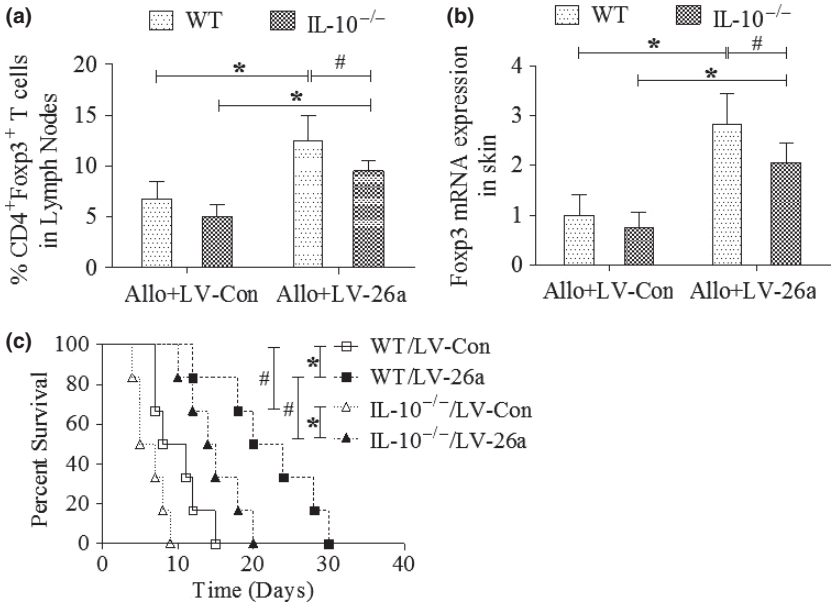

Figure 5 Mir-26a promotes Tregs expansion independent of IL-10 expression. (a) The proportions of $CD4^+Foxp3^+$ Tregs among $CD4^+$ T cells in draining lymph nodes of WT or IL-$10^{-/-}$ mice were analyzed by FACS; (b) Foxp3 mRNA expression in skin allograft of WT or IL-$10^{-/-}$ mice was analyzed by Q-PCR; (c) indicating skin allograft survival; $^*P < 0.01$, $^\#P < 0.05$, $n = 6$.

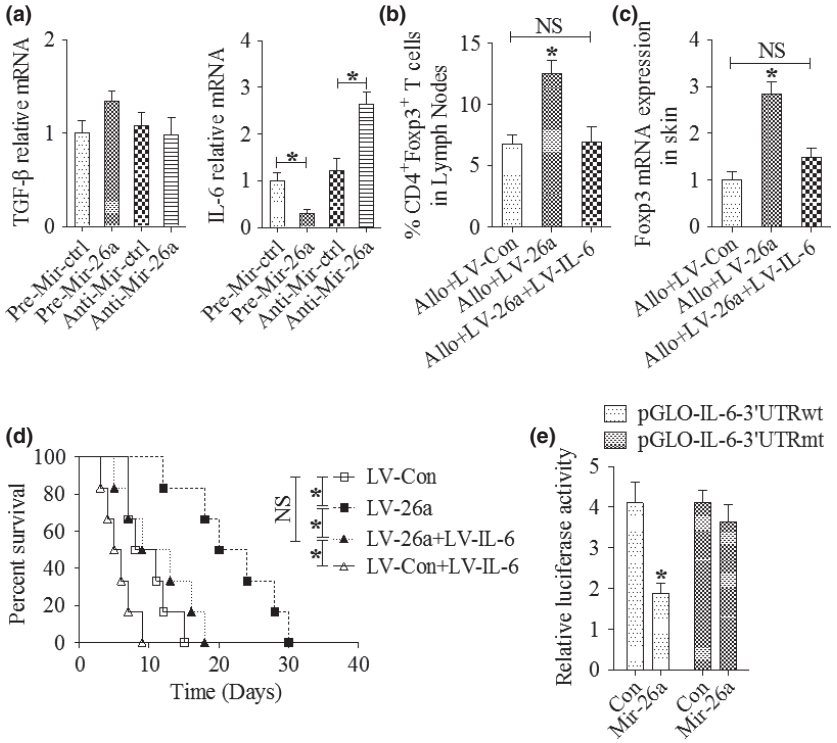

Figure 6 Mir-26a promotes Tregs expansion through repression of IL-6 expression. (a) Pre-Mir-ctrl, pre-Mir-26a, anti-Mir-ctrl, and anti-Mir-26a were transfected into $CD4^+$ T cells, which were then activated and polarized. The TGF-β and IL-6 mRNA levels were analyzed by Q-PCR 3 days after transfection and activation; (b) the proportions of $CD4^+Foxp3^+$ Tregs among $CD4^+$ T cells in draining lymph nodes were analyzed by FACS; (c) Foxp3 mRNA expression in skin allograft was analyzed by Q-PCR; (d) indicating skin allograft survival; (e) luciferase activity assay of HEk293 cells at 36 h after cotransfection with Mir-26a and pGLO-IL-6-3′ UTR or pGLO-IL-6-3′ UTR mutant. $^*P < 0.01$, $n = 6$. NS indicates no significant difference.

overexpression. Based on the important role of Tregs in inducing transplant tolerance, over expression of Mir-26a by infection of vectors encoding pre-Mir-26a may be exploited in clinical in future.

The differentiation of Treg cells may be linked to the differentiation of Th17 cells, depending on the cytokine milieu [22]. It has become clear that IL-6 plays a pivotal role in the dichotomous differentiation programs of Th17 and induced Tregs. Under situations with low concentration of TGF-β, IL-6 drives the development of Th17 cells at the expense of Tregs, whereas high concentration of TGF-β in the absence of proinflammatory cytokines (such as IL-6) induces the differentiation of Tregs from naive T cells [12,23]. Mir-26a is a kind of highly conserved short RNA that regulates diverse cellular processes by binding to the 3′ untranslated region (3′ UTR) of target messenger RNAs. In our current study, we found that overexpression of Mir-26a decreased the luciferase reporter activity of wild-type 3′ UTR but not mutant 3′ UTR of IL-6. IL-6 overexpression led to significant suppression of the Mir-26a-induced upregulation of Foxp3. All of these were consistent with previous paper demonstrating that Mir-26a inhibited tumor growth and metastasis in part by suppressing IL-6 [24,25].

Tregs are associated with the secretion of IL-10 in inflammatory responses [26] and inhibit the secretion of proinflammatory cytokines, such as TNF and IL-6 [27,28]. It has been reported that IL-10 is crucial for tolerance induction and is mainly expressed by Tregs and Kupffer cells, and the depletion of Tregs resulted in reduced plasma IL-10 levels [29]. In our current study, the Foxp3 mRNA expression and skin allograft survival were decreased in LV-26a-infected IL-10 deficiency mice when compared with LV-26a-infected wild-type mice, and LV-26a also induced Tregs expansion and prolonged skin allograft survival in IL-10 knockout mice as well as wild-type mice. All of these indicated that IL-10 is dispensable in the LV-26a-mediated expansion of Tregs and protective effects for skin allograft.

In summary, our data document an immune regulatory role for Mir-26a in the skin transplantation. Mir-26a overexpression results in prolonged skin allograft survival and promoted Tregs expansion in part by inhibition of IL-6. Although further investigations are needed to fully clarify the precise molecular and cellular mechanism involved in the immunoregulation, Mir-26a may be a novel therapeutic strategy to protect skin allograft.

Authorship

FX, JC and ZZ: participated in research design. FX, JC, ZZ, QH, TM: participated in the performance of the study. QH and TM: participated in the writing of the manuscript.

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